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HtrA1-dependent proteolysis of TGF-β controls both neuronal maturation and developmental survival

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Transforming growth factor-β (TGF-β) signalling controls a number of cerebral functions and dysfunctions including synaptogenesis, amyloid-β accumulation, apoptosis and excitotoxicity. Using cultured cortical neurons prepared from either wild type or transgenic mice overexpressing a TGF-β-responsive luciferase reporter gene (SBE-Luc), we demonstrated a progressive loss of TGF-β signalling during neuronal maturation and survival. Moreover, we showed that neurons exhibit increasing amounts of the serine protease HtrA1 (high temperature responsive antigen 1) and corresponding cleavage products during both *in vitro* neuronal maturation and brain development. In parallel of its ability to promote degradation of TGF-β1, we demonstrated that blockage of the proteolytic activity of HtrA1 leads to a restoration of TGF-β signalling, subsequent overexpression of the serpin type -1 plasminogen activator inhibitor (PAI-1) and neuronal death. Altogether, we propose that the balance between HtrA1 and TGF-β could be one of the critical events controlling both neuronal maturation and developmental survival.
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Transforming growth factor-β controls a plethora of neuronal and glial functions throughout life, under normal or pathological conditions including the functional neuronal connectivity,[1] apoptotic pathways,[2,3] glutamatergic signalling[4] and amyloid-β turnover.[5] TGF-β isoforms elicit their cell type-specific responses through the ligand-induced formation of a heteromeric receptor complex between the serine/threonine kinases TβR-I and TβR-II: the type II receptor binds TGF-β by its own, then recruits the type I receptor, allowing the transphosphorylation-mediated activation of TβR-I. The subsequent activation of the Smad (signalling mother against decapentaplegic peptide) transcription factor cascade thus regulates the transcription of key target genes.[6] Historically, Smads were thought to be the main substrates and the key signal transducers of activated TGF-β receptors. However, an ever increasing complexity in the canonical TGF-β intracellular cascade emerges, with the discovery of cross-talks with other signalling pathways such as mitogen-activated protein kinases or Wnt and the description of multiple regulatory factors, including Ski and SnoN or processes such as ubiquitination.[1,7]

The bacterial serine-protease HtrA, also known as DegP, is a heat shock-induced envelope-associated serine protease.[8] Its main role is to recognize and degrade misfolded proteins in the periplasm, combining a dual activity of chaperone and protease.[9] Four human homologues of HtrA have been identified and named respectively human HtrA1 (PRSS11 or L56),[10] HtrA2 (Omi),[11] HtrA3 (PRSP) and HtrA4. HtrA2 is localized at the mitochondrial membrane and is involved in apoptosis.[12] HtrA1, HtrA3 and HtrA4 are secreted proteases. Ubiquitously expressed in normal human adult tissues,[13] HtrA1 contains in addition to a highly conserved protease domain (trypsin like serine protease domain), an insulin growth factor-binding domain, a kazal type S protease inhibitor domain followed by one or two post-synaptic disc-large zona domains and a follistatin-like domain.[10] There is increasing evidences that HtrA1 regulates several physiological and pathological processes including tumour development,[14] Alzheimer's disease (AD),[15] placentation,[16] age-related macular degeneration[17] and osteoarthritis.[10] However, the mechanism(s) by which it regulates these processes has(ve) not been fully elucidated. Considering its subcellular distribution, either found as a secreted protein or in the cytoplasm of the cells,[16] HtrA1 could act on different targets including extra-cellular matrix proteins. Interestingly, both HtrA1 and HtrA3 isoforms have been reported to inhibit TGF-β signalling during development[18,19] through a proteolytically-dependent interaction with ligands of the TGF-β family. In parallel, the analysis of HtrA1 protein sequence has revealed that the amino-terminal region of human HtrA1 is closely related to mac25, a putative growth suppressor considered to act as an activated follistatin.[20] It is well known that follistatin plays an important role in the negative regulation of TGF-β family members such as BMP4, Gdf5, TGF-βs and activin, glycoproteins belonging to the TGF-β superfamily.[21]

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In the present study, we hypothesized that the serine protease HtrA1 could play a critical role in controlling TGF-β-dependent neuronal signalling and neuronal survival.

Results

A loss of TGF-β signalling occurs with *in vitro* neuronal maturation. mRNA levels for NMDA receptor subunits, synaptophysin, synapsin and PSD95 (post synaptic density protein of 95 kDa) were studied in primary cultures of cortical neurons maintained from 2 to 14 days *in vitro* (DIV). With DIV, cultured neurons showed increased levels of mRNAs encoding for NMDA receptor subunits 1, 2A, 2B, 2D (Figure 1a), for synapsin, synaptophysin and PSD95 (Figure 1b), all previously characterized[22,23] as markers of neuronal differentiation and functionality. Then, TGF-β signalling was investigated in sister cultures obtained as described in the methods section, from transgenic mice overexpressing the previously described luciferase reporter gene under the control of the TGF-β-specific Smad-binding element (SBE), CAGA box[24] and maintained 2, 5, 7 and 14 DIV. Both cultured neurons and astrocytes were treated in the presence of recombinant TGF-β1 (1 ng/ml, 24 h) and luciferase reporter activity measured. Although primary cultured astrocytes are responsive to TGF-β whatever the duration of *in vitro* maturation studied (up to fivefold induction) (Figure 2a), neurons lose their responsiveness to TGF-β between 5 and 7 days *in vitro* (Figure 2b). This effect is maintained at least until 14 DIV. To note, immunoblotting performed against the astrocytic marker (glial fibrillaric acidic protein) shows that our primary cultures of neurons are not contaminated with significant amounts of astrocytes (Figure 2c), demonstrating the neuronal specificity of the results obtained. As TGF-β signalling was previously demonstrated to control the expression of the serpin plasminogen activator inhibitor (PAI-1),[4] mRNA levels for PAI-1 were determined in control conditions at the different stages *in vitro* by using quantitative RT-PCR. As previously reported,[25] whatever *in vitro* stage, astrocytes exhibit high levels of mRNA encoding for PAI-1 (data not shown). In contrast (Figure 3a), although neurons maintained 2 days *in vitro* display high levels of mRNA for PAI-1, this expression is dramatically decreased with days *in vitro* with a reduction of up to 50% between 5–14 DIV. In parallel, the expression of the other serpins related to tPA, neuroserpin and protease nexin 1 (PN1, slight increase) and the expression of tPA failed to be influenced by the stage of *in vitro* development (Figure 3a). In addition, establishing the link between TGF-β signalling and PAI-1 mRNA levels, our data show that PAI-1

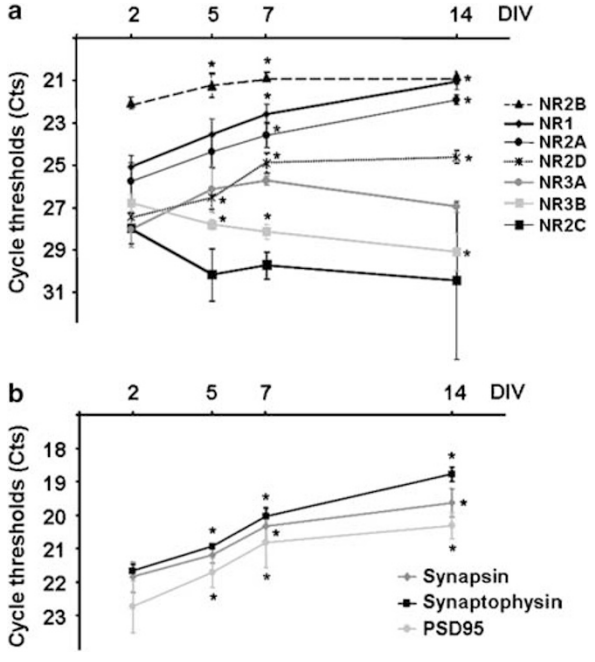

Figure 1 Increased expression of a set of neuronal markers with maturation. (**a**) mRNA levels of NMDA receptor subunits (NR1 to NR3B) in cultured neurons at 2, 5, 7 and 14 DIV ($N=3$, $n=9$). (**b**) mRNA levels for postsynaptic markers (Synapsin, Synaptophysin, PSD95) in cultured neurons at 2, 5, 7 and 14 DIV ($N=3$, $n=9$). Relative levels of mRNA expression were measured by quantitative PCR. Results were computed in Cycle thresholds (C_{ts}). Points represent mean values ± S.D. *Indicates significantly different from the 2 DIV value ($^*P<0.05$) by one way Kruskall–Wallis test followed by Mann–Whitney *post hoc* test

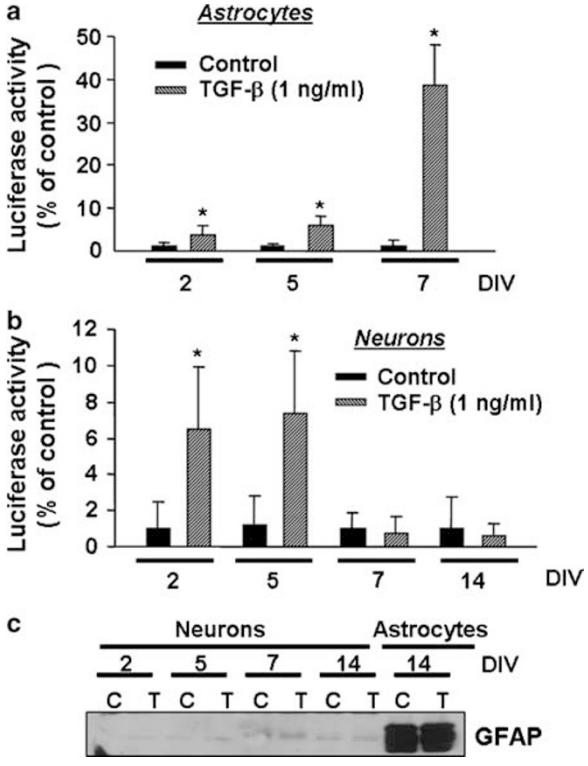

Figure 2 Neurons lose their responsiveness to TGF-β. (**a** and **b**) Cultured neurons and astrocytes obtained from SBE-luc transgenic mice displaying a luciferase reporter gene under the control of a Smad binding element: SBE[24] were treated at 2, 5, 7 ($n=12$) and 14 DIV ($n=4$) for neurons and 2, 5 and 7 DIV ($n=6$) for astrocytes in the presence of TGF-β1 at 1 ng/ml for 24 h. Luciferase activity from cell lysates was quantified by luminescence (mean ± S.D.) using the luciferase system kit, as described by the manufacturer. *Indicates significantly different from control ($^*P<0.05$) by one way Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**c**) Neurons at 2, 5, 7 and 14 DIV and astrocytes at 14 DIV were treated or not in the presence of TGF-β1 at 1 ng/ml for 24 h. Immunoblots were revealed with an antibody raised against mouse GFAP ($N=3$)

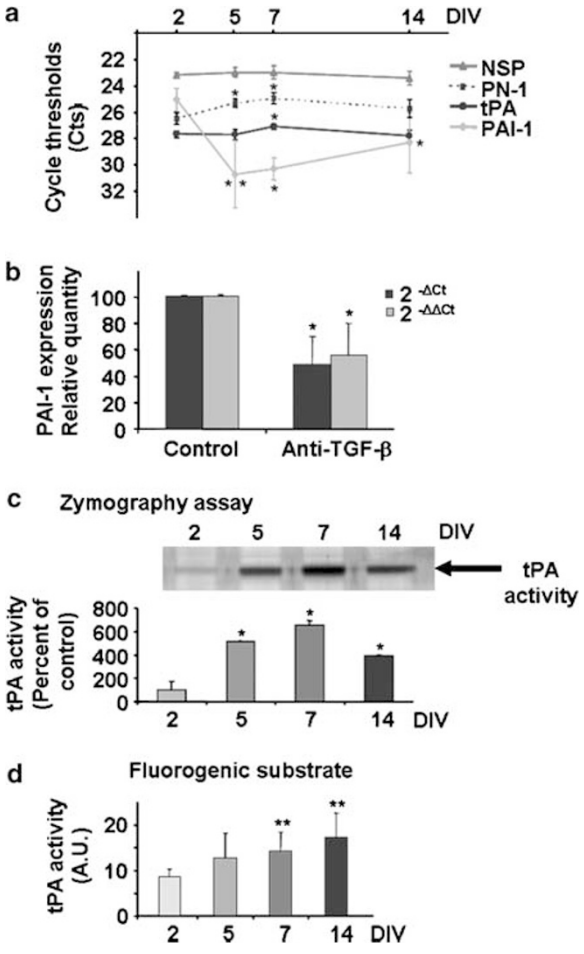

Figure 3 Neurons lose PAI-1 expression with DIV. (**a**) Evaluation of tPA and its serpins (protease nexine 1 (PN-1), plasminogen activator inhibitor (PAI-1) and neuroserpin (NSP)) mRNA levels during neuronal *in vitro* maturation ($N=6$, $n=18$). Relative levels of mRNA expression were measured by quantitative PCR. Points represent mean values ± S.D. *Indicates significantly different from the 2 DIV value (*$P<0.05$, **$P<0.01$). (**b**) Evaluation by real time PCR of the mRNA levels for PAI-1 in cultured neurons treated at 2 DIV with a blocking antibody raised against TGF-β at 5 μg/ml for 48 h (Pan-specific TGF-β antibody, R&D Systems). Results were computed with the two following formulas: black bars represent $2^{-\Delta Ct}(=2^{-(\Delta Ct \text{ of PAI}-1)})$ and grey bars represent $2^{-\Delta\Delta Ct}(=2^{-(\Delta Ct \text{ of PAI}-1-\Delta Ct \text{ of cyclophilin})})$. Bars represent mean values ± S.D. *Indicates significantly different from control value (*$P<0.05$) by one way Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**c**) Zymography analysis of tPA proteolytic activity was performed on proteins extracted from cultured neurons at 2, 5, 7 and 14 DIV old with densitometric analysis. Data are represented as mean ± S.D. ($N=3$) relative to 2 DIV cultured neurons; (*$P<0.05$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**d**) tPA activity is increased with DIV. tPA activity was measured by using a fluorogenic substrate (XF444) on medium obtained from cultured neurons at 2, 5, 7 and 14 DIV old. Data are represented as mean ± S.D. ($N=3$, $n=6$) relative to 2 DIV cultured neurons (*$P<0.05$)

expression is significantly reduced by a 48 h treatment of 2 DIV cultured neurons in the presence of a previously characterized blocking antibody raised against TGF-β (Figure 3b). Then, the endogenous proteolytic activity of tPA contained either in the neuronal monolayer (Figure 3c) or in corresponding bathing media (Figure 3d) was measured as described in the Materials and methods section. We show that lowering of PAI-1 expression is, as expected, associated with an enhanced proteolytic activity of tPA.

Upregulation of the expression of the serine protease, HtrA1, occurs both during *in vitro* neuronal maturation and *in vivo* brain development. Microarray analyses comparing gene expression in primary cultures of neurons maintained for 2 *versus* 15 DIV revealed that among around 10 000 genes analyzed, the expression of some of them was significantly influenced by the stage of *in vitro* maturation (See GEO database under the accession number GSE11162). Interestingly, while selecting serine proteases as a keyword for analyzing the microarray data, we found that the high temperature responsive antigen 1 (HtrA1 or PRSS11) gene was upregulated with DIV. This was then confirmed by quantitative PCR in human and murine neuronal cultures.

Thus, we have postulated that an overexpression of the previously characterized antagonist of TGF-β signalling, HtrA1, could control the loss of neuronal TGF-β signalling observed with days *in vitro*. By using quantitative RT-PCR, although mRNA expression for both TGF-β receptors (TβR-I and TβR-II) were not modulated (data not shown), overexpression of HtrA1 was confirmed in primary cultures of cortical neurons from mice (Figure 4a) with an upregulation of its expression with days *in vitro* (up to twofold increase). Quantitative RT-PCR performed in parallel from mRNA harvested in primary cultures of mouse cortical neurons at 2, 5, 7 and 14 DIV revealed no modification of the expression of HtrA2, 3 and 4 (Figure 4a). Neuronal overexpression of HtrA1 with days *in vitro* was also confirmed at the protein level by immunoblotting performed from the cell monolayer revealed with an antibody raised against HtrA1 (Figure 4b). Immunoblotting showed an enhanced positive processing/cleavage of HtrA1 with days *in vitro*. Parallel experiments were performed *in vivo*. mRNA levels for both HtrA1 and PAI-1 were measured from brain cortex of mice at different stages of development (E16 to adults). As shown *in vitro*, we can observe a dramatic increase of the mRNA levels encoding for HtrA1 in animals from E20 to adults (Figure 4c), with an associated decrease in the mRNA levels encoding for PAI-1 (Figure 4d). It is interesting to note, that the overexpression of HtrA1 appeared before the decrease of PAI-1 (E20 *versus* P1). Similarly, immunoblotting raised against HtrA1 revealed an increased level of HtrA1 associated with an increased processing/cleavage of the protein HtrA1 during brain development (Figure 4e). Immunohistochemistry performed in animals from E16 to P1 showed positive staining for HtrA1 during brain development in neurogenic areas including the ventricular zones (Figure 5a–c). In adult brains, positive immunostainings for HtrA1 were observed mainly in cortical areas both in glial cells (GFAP stained; Figure 5h) and neurons (either β-III tubulin (Tuj 1) or NeuN-stained; Figure 5e–g). Neuronal HtrA1 immunostainings displayed mainly a membrane-associated labelling.

HtrA1 controls neuronal TGF-β signalling and subsequent neuronal survival. To further investigate the relationship between TGF-β and HtrA1 and their ability to control the expression of the serpin PAI-1 in neurons, primary cultures of cortical neurons were treated with the previously characterized HtrA1 inhibitor, NVP-LBG976. After 2 days of treatment, the reduction of PAI-1 expression (mRNA)

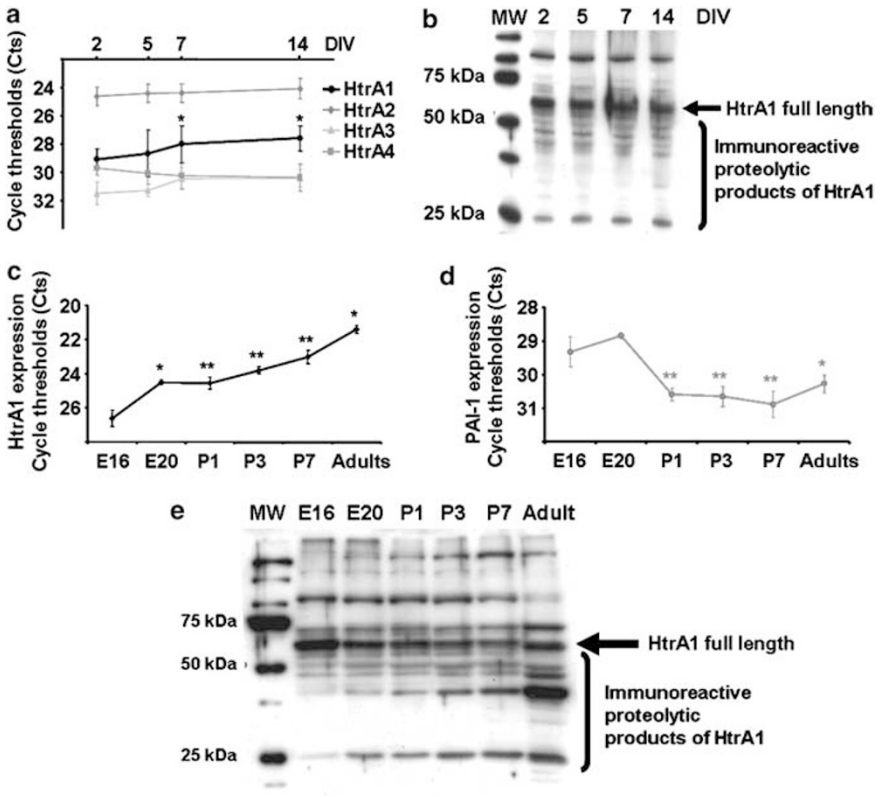

Figure 4 Expression of HtrA members and PAI-1 with neuronal maturation and *in vivo* brain development. (**a**) Evaluation by real time PCR of the mRNA levels for HtrA1, HtrA2, HtrA3 and HtrA4 in cultured neurons at 2, 5, 7 and 14 DIV ($N=3$, $n=9$). Results were computed in C_{ts}. Points represent mean values ± S.D. *Indicates significantly different from the 2 DIV value (*$P<0.05$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**b**) Immunoblot raised against HtrA1 was performed on proteins extracted from cultured neurons at 2, 5, 7 and 14 DIV old ($N=4$). (**c** and **d**) Evaluation by real time PCR of the mRNA levels for HtrA1 and PAI-1 in cerebral cortex of Swiss mice from E16 to adults (E16 $N=6$, E20 $N=3$, P1 $N=6$, P3 $N=6$, P7 $N=6$ and adults $N=4$). Results were computed in C_{ts}. Points represent mean values ± S.D. *Indicates significantly different from the E16 value (*$P<0.05$, **$P<0.01$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**e**) Immunoblot raised against HtrA1 was performed on proteins extracted from cerebral cortex of Swiss mice from E16 to adults ($N=3$)

previously observed with days *in vitro* was restored. Indeed, although PAI-1 mRNA levels were decreased with DIV in control neurons, blockage of the proteolytic activity of HtrA1 increased PAI-1 mRNA levels (up to 100% at 24 h, up to 400% at 48 h, up to 400% at 72 h and up to 200% at 96 h (Figure 6a). Accordingly, treatment of neurons with the HtrA1 inhibitor led to an associated decrease of the endogenous proteolytic activity of tPA as estimated by fluorogenic and zymography assays (Figure 6b and c respectively). Then, the possible involvement of both HtrA1 and TGF-β signalling in the control of neuronal survival was investigated. Primary cultures of cortical neurons maintained for 2 days *in vitro* were treated with the HtrA1 inhibitor (NVP-LBG976, 6 μM) for 5 days (from 2 to 7 DIV) prior to estimation of neuronal survival. Blockage of the proteolytic activity of HtrA1 led to a significant neuronal death, an effect prevented by either the co-application of an anti- TGF-β blocking antibody or recombinant tPA while by themselves these treatments did not affect cell survival (Figure 6d). Interestingly, as it was reported that HtrA1 could inhibit TGF-β signalling through a proteolytically-dependent interaction,[19] the ability of purified recombinant HtrA1 to cleave recombinant TGF-β1 was tested (Figure 7). Our data clearly show that co-incubation of proteolytically active HtrA1 with recombinant TGF-β1 leads to a dramatic degradation of the cytokine. Altogether, these data demonstrate that progressive loss of neuronal TGF-β signalling is a consequence of an overexpression and proteolytic process of neuronal HtrA1, an effect leading to a reduced PAI-1 expression, an increased proteolytic activity of tPA and subsequent neuronal survival and maturation (Figure 8).

Discussion

Although the cytoplasmic HtrA1 was involved in chemotherapy-induced apoptosis,[26] other reports supposed that HtrA1 could also be secreted and act in the extracellular space. Indeed, HtrA1 has been detected in the synovial fluid of osteoarthritic and rheumatoid arthritic patients[27] and has been described to be secreted by astrocytes.[15] Here, we evidence that cultured neurons exhibit both mRNAs and proteins for HtrA1. Moreover, we show that HtrA1 expression is associated with neurogenic areas during brain development and with both glial cells and neurons in the adult brains. Although the mechanisms of HtrA1 actions remain largely unknown, HtrA1 was reported to inhibit TGF-β family signalling.[19] Indeed, experiments using series of deletion mutants for HtrA1 have indicated that HtrA1 and possibly HtrA3 could bind some TGF-β family members such as BMP4, Gdf5, TGF-βs and activin[18,19] leading to a proteolytically dependent

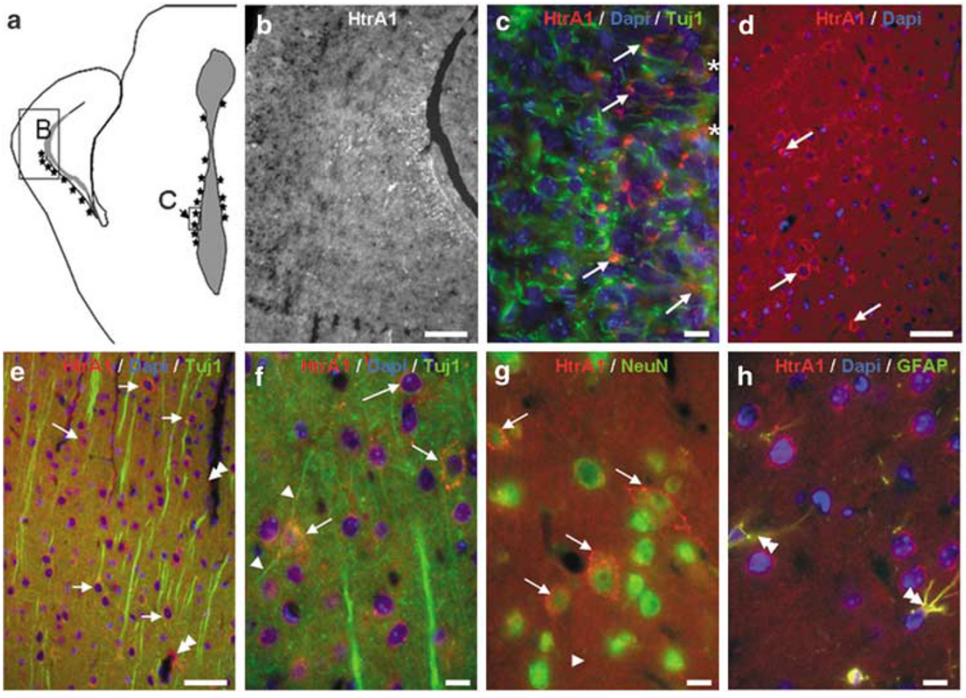

Figure 5 Immunohistochemical detection of HtrA1 in E16 embryo (**a**–**c**) or adult cortices (**d**–**h**). (**a**) Schematic view of a coronal section showing neurogenic areas expressing HtrA1 immunoreactivity (stars). At this stage, these areas are characterized by an intense immunostaining for HtrA1 (arrows in **b** or **c**). At high magnification (**d**), HtrA1 immunoreactivity reveals large punctuation associated to cells of the ventricular zone (stars show the third ventricle). Coronal sections of adult cortices (**d**–**h**) display a large distribution of HtrA1 immunoreactivity. In the adult cortex, HtrA1 immunoreactivity is associated to neuronal cell bodies (arrows in **d**–**g**), astrocytes and astrocytic endfeets (double arrowheads in **e** and **h**). The neuronal expression of HtrA1 is revealed by double immunostainings in cortical neurons (arrows in **e**–**f**) revealed either by Tuj 1 (FITC in **e** and **f**) or by NeuN immunoreactivity (FITC in **g**). The astrocytic localization of HtrA1 immunoreactivity is shown in (**h**) (yellow labeling) in the merged image of a double immunostaining against GFAP (FITC) and HtrA1 (TRITC) in neuronal cell bodies. Scale bars represent 200 μm for (**b**) 50 μm for (**d**, **e**) and 10 μm for (**c**, **f**, **g** and **h**)

inhibition of TGF-β-related signalling. Accordingly, we provide here, the demonstration that regulation of the proteolytic activity of endogenous HtrA1-controlled neuronal TGF-β signalling. Biological actions of TGF-βs are mediated by high affinity serine-threonine kinase receptors (TβR-I and TβR-II) and subsequent activation of the Smad-dependent signalling cascade leading to the transcriptional regulation of a set of gene-promoters containing the Smad-binding element (SBE) also named CAGA box.[28] Using primary cultures of cortical neurons and astrocytes from transgenic mice displaying the SBE-luciferase reported gene, known to reveal cerebral TGF-β signalling,[24] we show that although neurons are responsive to TGF-β signalling when maintained for a short period *in vitro*, they lose TGF-β responsiveness with *in vitro* differentiation/maturation. These data are in agreement with the low endogenous TGF-β signalling observed in adult brains under physiological conditions, a signal dramatically increased following brain injury.[29] In parallel, our *in vivo* data show an overprocessing (mRNA and protein levels) of HtrA1 during brain development (E16 to adult brains) associated with a reduced expression of the TGF-β-dependent regulated serpin, PAI-1. TGF-β has been reported to play critical and complex functions and sometimes opposite effects in the central nervous system both during brain development and in the injured adult brains. For example, TGF-βs control synaptic growth and neuronal differentiation. Mice with reduced TGF-β1 mRNA levels showed an increased susceptibility to age- and excitotoxin-induced brain injury.[30] During AD, TGF-β has been suggested to play critical roles. However, conflicting data have been reported, for instance regarding TGF-β levels in AD. Indeed, although some authors have found decreased serum levels of the cytokine,[31] others have reported no difference between AD patients and age-matched controls and no association between serum TGF-β levels and AD.[32] Similarly, although it has been proposed that AD is accompanied by increased TGF-β levels within the brain parenchyma,[31] a recent report suggests that TGF-β signalling might be downregulated due to a reduced expression of its TβR-II receptor.[33] Similarly, conflicting data have been published regarding the potential role of altered TGF-β levels in AD. For instance, in human amyloid precursor protein (hAPP) transgenic mice, a moderate increase in astrocytic expression of TGF-β1 was proposed to lead to a reduced Aβ accumulation because of a TGF-β-dependent phagocytosis of Aβ by microglia.[34] At the opposite, a deficiency in neuronal TGF-β signalling was reported to promote neurodegeneration and Alzheimer's pathology.[33] Interestingly, HtrA1 was also directly involved in the β-amyloid pathway, in which HtrA1 colocalizes with β-amyloid deposits in human brain samples and reduces Aβ accumulation in astrocytes cell culture supernatants.[15]

In parallel, we have previously demonstrated that TGF-β treatment in both murine and human cultured astrocytes leads to an increased transcription of APP and subsequent accumulation of Aβ.[5] Although brain TGF-β levels are highly increased following cerebral ischemia,[29] TGF-β was reported to protect hippocampal, cerebellar and cortical[35] neurons

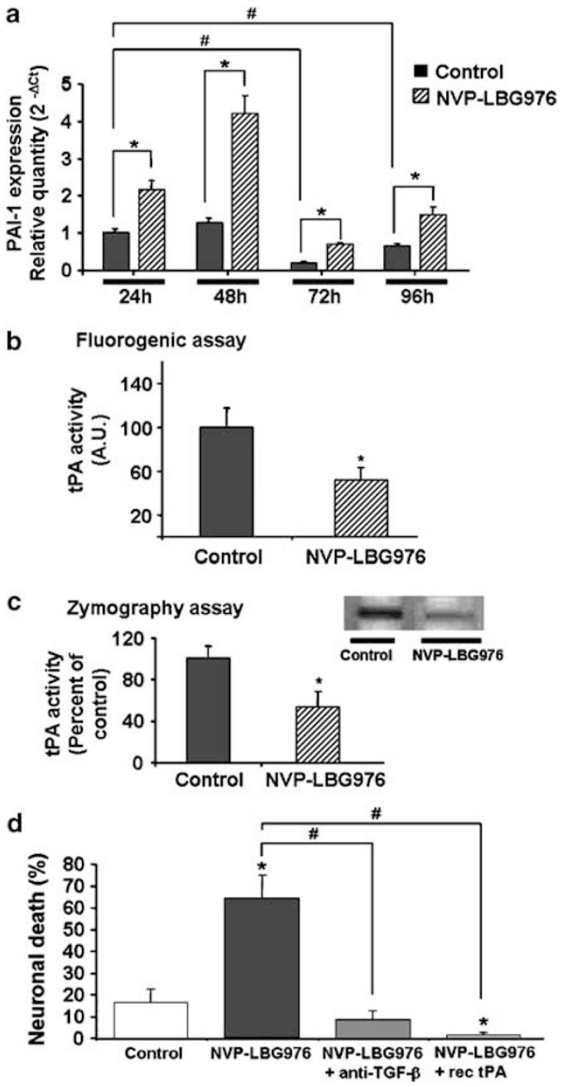

Figure 6 Effect of the HtrA1 inhibitor: NVP-LBG976 on cultured neurons. (**a**) Evaluation by real time PCR of the mRNA levels for HtrA1 in cultured neurons treated at 2 DIV with NVP-LBG976 (6 μM) for 24, 48, 72 and 96 h ($N=3$, $n=9$). Data are represented relative to control with the $2^{-\Delta Ct}$ method. Bars represent mean values ± S.D. *Indicates significantly different from control and #Indicates significantly different from the 24 h control (*$P<0.05$, #$P<0.05$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**b**) tPA activity was measured by using a fluorogenic substrate (XF444), from bathing media of cultured neurons at 2 DIV treated or not with NVP-LBG976 (6 μM) for 48 h. Data are represented as mean ± S.D. ($N=3$, $n=6$) relative to control. (**c**) tPA activity is decreased with NVP-LBG976. Zymography assays for the tPA proteolytic activity were performed from protein extracts of cultured neurons at 2 DIV treated or not with NVP-LBG976 (6 μM) for 48 h. Data are represented as mean ± S.D. ($N=3$) relative to control; (*$P<0.05$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test. (**d**) Pure cortical neuronal cultures were treated at 2 DIV with NVP-LBG976 (6 μM), an antibody raised against TGF-β (5 μg/ml) and recombinant tPA (20 μg/ml) for 5 days. At 7 DIV, the percentage of neuronal death was assessed by trypan blue dye staining. Bars represent mean ± S.E.M. ($N=3$, $n=9$). *Indicates significantly different from control (*$P<0.05$); #Indicates significantly different from NVP-LBG976 (#$P<0.05$); Kruskall–Wallis test followed by Mann–Whitney *post hoc* test

against *N*-methyl-D-aspartate (NMDA) toxicity. This neuroprotective activity requires the obligatory induction of the synthesis and release of the type 1 plasminogen activator inhibitor (PAI-1) by astrocytes upon activation of TGF-β receptors and through a recruitment of Smad3.[4] Here, we provide evidence that neurons lose both TGF-β signalling and PAI-1 expression with neuronal differentiation, a phenomenon associated to an overexpression of HtrA1. Interestingly, in agreement with previous reports,[19] we provide *in vitro* evidence that proteolytically active HtrA1 is capable to mediate proteolysis of TGF-β1 as an explanation of its ability to prevent TGF-β signalling. In addition, our data demonstrate that an inhibitor of the proteolytic activity of HtrA1 rescued neuronal expression of PAI-1 *in vitro*. It has previously been demonstrated that the serine protease, tissue type plasminogen activator (tPA), initially characterized for its capacity to induce fibrinolysis, was also expressed within the cerebral parenchyma to play critical functions including control of memory, learning processes[36] and excitotoxic neuronal death in the injured brain.[37] Here, we demonstrate that regulation of neuronal TGF-β signalling by HtrA1, controls both PAI-1

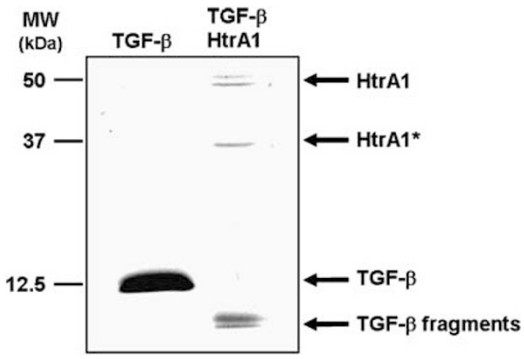

Figure 7 Degradation of TGF-β1 by HtrA1. Three micrograms of purified TGF-β1 were incubated with 0.3 μg of HtrA1 in 50 mM Tris-HCl pH 8 overnight at 37°C. As a control, TGF-β (lane 1) was incubated separately overnight at 37°C. Samples were loaded on a SDS/PAGE. The gel was stained with Coomassie Blue. HtrA1* represents autoproteolysis of HtrA1. (Representative of three independent experiments)

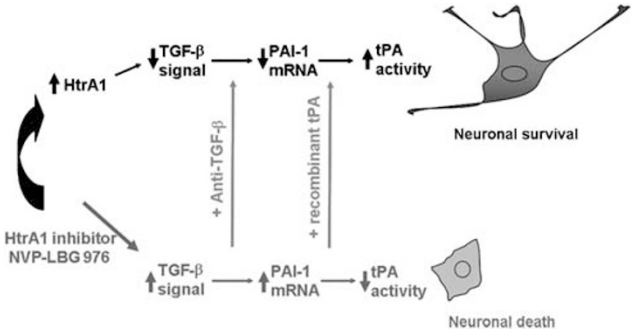

Figure 8 Schematic hypothesis of HtrA1 mechanism of action. We have observed that neurons have lost TGF-β response during *in vitro* differentiation, a phenomenon leading to a reduced expression of the serpin, PAI-1 and subsequent increase of the tPA-dependent proteolytic activity. Interestingly, loss of neuronal TGF-β signalling is dependent on the increased expression and proteolytic activity of the serine protease HtrA1. Indeed, an inhibitor of the proteolytic activity of HtrA1 rescues both neuronal TGF-β signalling, PAI-1 expression and reduced tPA activity. In addition, we evidence that loss of neuronal TGF-β signalling is critical for neuronal survival. Indeed, neuronal death when initiated in the presence of the HtrA1 inhibitor is prevented by co-addition of either a blocking antibody for TGF-β or recombinant tPA

expression and subsequent endogenous tPA proteolytic activity. Interestingly, our present data reveal that the balance between HtrA1 and TGF-β signalling is a critical event for the control of neuronal survival. Indeed, blockage of the proteolytic activity of HtrA1 promotes neuronal death, a phenomenon blocked by the co-application of either a blocking antibody for TGF-β signalling or addition of recombinant tPA (Figure 6d).

A number of HtrA1 extracellular targets such as the C-propeptides of fibril-forming types I, II and III procollagen,[38] fibronectin[27] and proteoglycans such as aggrecan have been identified to play important functions in extracellular matrix remodeling. Our data evidence that HtrA1 controls neuronal TGF-β signalling as a critical event to maintain developmental neuronal survival. HtrA1 could thus be now considered as a 'cytokine-like molecule' of the central nervous system, involved in neuronal maturation during brain development and as a new target for neuroprotection or brain recovery.

Materials and Methods

Materials. Horse serum, fetal bovine serum and laminin were purchased from Invitrogen. Human rt-PA (Actilyse) was purchased from Boerhinger Ingelheim (Paris, France). The blocking antibody raised against TGF-β was purchased from R&D Systems (Lille, France). α-casein was obtained from ICN Biomedicals (Aurore, OH, USA), and human Lys-plasminogen was purchased from Calbiochem. tPA substrate spectrozyme XF-444 was purchased from American Diagnostica (France). HtrA1 inhibitor (NVP-LBG976) was provided by Novartis (Basel, Suisse). All the other chemicals were obtained from Sigma.

Animals. Transgenic mice overexpressing a Smad-binding element luciferase reporter construct (SBE-Luc mice) were provided by T Wyss Coray (Department of neurology and neurological sciences, Stanford, USA). SBE-Luc mice were generated with the SBE-luc plasmid[39] on a FVB/N or (SJL/J $\times$ C57BL/6J)F_1 genetic background using standard procedures.[24]

Cortical proteins and mRNA extracts were obtained from Swiss mice at different ages including 16- and 20-day-old embryos (E16, E20), 1-, 3- and 7-day-old pups (P1, P3 and P7) and 8-month-old adult mice.

Primary cultures of cortical neurons and astrocytes. Cultured neurons or astrocytes were prepared from mouse embryos (E15–E16) and pups (P1-3) respectively.[40] Cell suspensions in Dulbecco's modified Eagle's medium (DMEM, Sigma) were seeded on plates coated with 0.1 mg/ml poly-D-lysine (Sigma) and 0.02 mg/ml laminin for neurons or 0.1 mg/ml poly-D-lysine for astrocytes. Cells were cultured in DMEM containing fetal bovine serum, horse serum (5% each for neurons and 10% each for astrocytes) and 2 mM glutamine (Sigma) and maintained at 37°C in a humidified 5% CO_2 atmosphere. For neuronal cultures, cytosine β-arabinoside furanoside (Ara-C, 10 μmol/l) was added after 3 days *in vitro* (DIV) to inhibit glial proliferation. Astrocyte cultures were washed every 3 days with phosphate-buffered saline (PBS) to eliminate microglia. Neuronal cultures were used after 2, 5, 7 or 14 days *in vitro* (DIV).

Reporter gene assay. Cultured neurons and astrocytes were obtained from the SBE-luciferase mice overexpressing a luciferase reporter gene under the control of a Smad-binding element, as described above. Luciferase activities were measured using a commercial luciferase® assay kit (Promega) as described by the manufacturer. Briefly, cultured neurons and astrocytes were treated at different stages of *in vitro* development with or without TGF-β (1 ng/ml) for 24 h. Treated cells were then exposed to a serum-free medium and luciferase activities were measured 24 h later.

Extraction of total RNA. Total RNAs were extracted from cultured cells or tissues by using the NucleoSpin® RNA II kit from Macherey-Nagel. The samples were lysed in RA1® buffer containing 1% of β-mercaptoethanol. After filtration of the lysates on Nucleospin filter units, total RNAs were purified on Nucleospin RNA II columns according to the manufacturer's instructions and eluted with RNase-free water.

Microarray analysis. Total RNA were extracted from primary cultures of human neurons maintained 2 and 15 days *in vitro*. For cultures and mRNA extractions, see Materials and methods described above. The quality of each RNA sample was then checked using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). 15 μg of total uRNA (Universal Human Reference RNA, Stratagene) and RNA obtained from neurons were converted into cDNA labeled with Cy3 or Cy5. The reverse transcription was performed with random hexamers overnight at 37°C in the presence of 10 mM DTT (Invitrogen), 1X First-Strand buffer (Invitrogen), 125 μM dNTP, 25 μM of fluorescent dye (Amersham) and 400 U of Superscript II (Invitrogen). Labeled cDNAs were then purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, and hybridized on HGMP arrays overnight at 42°C in 50% Formamide, 4 $\times$ SSC, 0.1% SDS and 5 $\times$ Denhardt's. After hybridization, the scans of HGMP arrays were performed by an Axon 4000B (Axon Instrument) and signal and background intensities were obtained using the GenepixPro 4.1 sofware (Axon Instrument).

Quantitative real-time RT-PCR. One microgram of total RNAs from each sample was reverse-transcribed using the Promega RT system (Promega, Charbonnieres, France; reverse transcription: 42°C for 1 h). Two primers were designed for each gene using the Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France). After design, primer alignments were performed with the Blast database to ensure the specificity of primers. Primer sequences used are available in Supplementary Table 1 (on Cell Death and Differentiation website). PCR solutions were prepared with RNase-free water containing primers and IQ SYBR Green Supermix (Biorad, Marnes-la-Coquette, France).

For PCR amplification, 20 μl of mix were added to 5 μl of reverse transcription reaction previously diluted (1:20). Two negative controls were performed during each experiment. In the first control, we used samples without reverse transcription as a template to control contamination of RNA with genomic DNA. In the second control, we used RNase-free water instead of cDNA to prove that qPCR mixes were not contaminated with DNA. Assays were run in triplicate on the iCycler iQ real-time PCR detection system (Bio-Rad). The amplification conditions were: Hot Goldstar enzyme activation, 95°C for 3 min; 50 cycles of PCR at 95°C, 15 s and 60°C, 1 min. The levels of expression of interest gene were computed as follows: relative mRNA expression $= 2^{-(Ct \text{ of gene of interest})}$ where C_t is the threshold cycle value. Expression of interest gene was also computed with respect to the mRNA expression level of the reference gene transcript using the second formula: relative mRNA expression $= 2^{-(Ct \text{ of gene of interest} - Ct \text{ of gene of reference})}$.

Protein extractions. Cells were lysed on ice in Tris-NaCl-Triton buffer containing 1% of protease inhibitor cocktail (Sigma) and 1% of phosphatase inhibitor (Sigma). To clear lysates, samples were centrifuged for 20 min (10 000 g) at 4°C. Proteins were quantified by using BCA protein assay (Pierce).

Immunoblotting. Protein samples (15 μl) were resolved on a 10% SDS-polyacryl-amide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% dried milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 and incubated with primary antibodies. After incubation with the corresponding secondary peroxidase-conjugated streptavidine reagent, proteins were visualized with an enhanced chemiluminescence ECL Plus immunoblotting detection system (Perkin Elmer-NEN, Paris, France).

tPA activity measurement. Two techniques were used to evaluate the proteolytic activity of tPA.

(1) Zymography assays were performed according to previously published methods.[41] Briefly, 15 μg of protein extracts were resolved on SDS-PAGE (12%) containing casein (1 mg/ml) and plasminogen (4.5 μg/ml). After migration, the gel was washed in a 2.5% triton solution and incubated for 3 h in a buffer containing glycine (100 mM) and EDTA (10 mM), pH 8.3. Finally, clear bands indicating proteolytic activity were visualized by Coomassie staining.

(2) tPA activity was measured by using a commercial fluorogenic substrate for human tPA (Spectrozyme® XF444). This substrate is a modified peptide corresponding to the cleavage site of tPA (methylsulfonyl-D-phenyl-glycyl-arginine-7-amino-4-methylcoumarin acetate). Briefly, 50 μl of conditioned medium were mixed with equal volume of tPA substrate (final concentration: 5 μM). Fluorescence (excitation: 360, emission: 440 nm) was monitored during 120 min and activity was next estimated by calculating the initial slope of the reaction. Results were expressed in arbitrary units (AU). The substrate was prepared according to manufacturer's instructions.

Immunohistofluorescence. Deeply anesthetized (500 mg/kg of choral hydrate) adult Swiss mice ($n=4$), were transcardially perfused with cold heparinized saline (15 ml) followed by 150 ml of fixative (sodium phosphate buffer 0.1 M. pH 7.4 containing 2% paraformaldehyde and 0.2% picric acid). Corresponding brains and embryonic brains (E16, $n=6$) were post-fixed or fixed, respectively, by immersion in the same fixative for 18 h. Tissues were frequently rinsed in a veronal buffer containing 20% sucrose before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryomicrotome-cut transversal sections (8–12 μm) were collected on gelatin-coated slides and stored at -80°C before processing.

Series of adjacent sections were immunostained with the rabbit polyclonal anti-HtrA1 (1:800[13]), associated, or not, with one of the following primary antibodies: mouse monoclonal anti-NeuN (1:500, Mab377, Chemicon), mouse monoclonal anti-GFAP (1:600, Ab-6I, Neomarkers) or mouse monoclonal anti-β III tubulin (Tuj 1) conjugated to fluorescein isothiocyanate (FITC) (1:300, ab25770 (TU-20), Abcam). Unstained primary antibodies were visualized with Fab'2 fragments of Donkey anti-rabbit IgG linked to tetramethyl rhodamine isothiocyanate (TRITC) or Fab'2 fragments of Donkey anti-mouse IgG linked to FITC (both 1:400, Jackson ImmunoResearch, West Grove, USA). Washed sections were coverslipped with antifade medium containing, or not, DAPI and images were digitally captured using a Leica DM6000 microscope-coupled coolsnap camera and visualized with Metavue software (Molecular Devices, USA).

Recombinant production and purification of HtrA1. Purified recombinant HtrA1 was produced in *Escherichia coli* and purified by using Ni^{2+}-NTA and ion exchange chromatography as previously described.[15]

Evaluation of neuronal death. Neuronal cultures were treated at 2 DIV with or without NVP-LBG976 (HtrA1 inhibitor) at 6 μM, in the presence or not of either an antibody raised against TGF-β isoforms (β1, β1.2, β2, β3 and β5) at 5 μg/ml or with recombinant tPA at 20 μg/ml for 5 days. Neuronal death was estimated by phase-contrast cell counting after staining with 0.4% Trypan blue dye. The percentage of neuronal death was estimated as the ratio of Trypan blue-positive neurons to the total number of neurons.

Statistical analysis. Data (mean $\pm$ S.D. and mean $\pm$ S.E.M.) were analyzed by the Kruskall–Wallis test, followed by *post hoc* comparisons, with the Mann–Whitney's test.

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