Imbalance of neurotrophin receptor isoforms TrkB-FL/TrkB-T1 induces neuronal death in excitotoxicity

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A better understanding of the mechanisms underlying neuronal death in cerebral ischemia is required for the development of stroke therapies. Here we analyze the contribution of the tropomyosin-related kinase B (TrkB) neurotrophin receptor to excitotoxicity, a primary pathological mechanism in ischemia, which is induced by overstimulation of glutamate receptors of the N-methyl-D-aspartate (NMDA) type. We demonstrate a significant modification of TrkB expression that is strongly associated with neurodegeneration in models of ischemia and in vitro excitotoxicity. Two mechanisms cooperate for TrkB dysregulation: (1) calpain-processing of full-length TrkB (TrkB-FL), high-affinity receptor for brain-derived neurotrophic factor, which produces a truncated protein lacking the tyrosine-kinase domain and strikingly similar to the inactive TrkB-T1 isoform and (2) reverse regulation of the mRNA of these isoforms. Collectively, excitotoxicity results in a decrease of TrkB-FL, the production of truncated TrkB-FL and the upregulation of TrkB-T1. A similar neuro-specific increase of the TrkB-T1 isoform is also observed in stroke patients. A lentivirus designed for both neuro-specific TrkB-T1 interference and increased TrkB-FL expression allows recovery of the TrkB-FL/TrkB-T1 balance and protects neurons from excitotoxic death. These data implicate a combination of TrkB-FL downregulation and TrkB-T1 upregulation as significant causes of neuronal death in excitotoxicity, and reveal novel targets for the design of stroke therapies.

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Stroke is a leading cause of death, dementia, and adult disability. The available therapies are insufficient to restrict brain damage; therefore, we need to understand the mechanisms underlying neuronal death in those regions surrounding the infarct. A primary pathological mechanism in ischemia is excitotoxicity, a form of neuronal death induced by overactivation of the N-methyl-D-aspartate (NMDA)-type of glutamate receptors (NMDARs), also associated with other acute disorders or neurodegenerative diseases.1 Nonetheless, efforts to inhibit NMDARs have generally failed, mainly due to the critical role these receptors have in neuronal survival,2,3 synaptic plasticity, learning, and memory.4 Pro-survival signaling initiated by synaptic NMDARs includes induction of antioxidant defenses,5 activation of extracellular signal-regulated kinase,6 and phosphorylation of the cAMP response element-binding protein (CREB), which results in the expression of brain-derived neurotrophic factor (BDNF)7,8 and its receptor, tropomyosin-related kinase B (TrkB).9,10 Pathological activation of NMDARs opposes these effects by coupling Ca2+-influx to mitochondrial dysfunction and neuronal death pathways.9,11 Additionally, NMDAR overactivation also disrupts critical survival pathways by uncoupling synaptic NMDARs from cytoskeletal proteins and pro-survival signaling. Transcriptional downregulation of the NMDAR subunit, GluN1,12 and calpain-processing of the GluN2 subunits13,14 and NMDAR-interacting proteins15,16 in excitotoxicity and ischemia have been demonstrated. Calpain is an effector of Ca2+-overload central to ischemia acting as a modulatory protease that transforms substrate stability, location, or activity.16 Processes downstream of the NMDAR overactivation, which are aberrant in stroke, might constitute novel therapeutic targets for reduction of neuronal death. We hypothesized that neurotrophin receptors, which are associated with NMDARs17,18 are modified by ischemia as other NMDAR-related proteins. Although neurotrophins have been proposed as therapeutic agents for stroke and excitotoxicity-associated diseases,19 the effect of ischemia on neurotrophin signaling is
largely unknown. These molecules are fundamental for the development of adult nervous system. Among them, BDNF has a major role through binding to its high-affinity tyrosine-kinase receptor, TrkB, and initiation of signaling cascades that regulate gene expression, neuronal survival, neurotransmitter release, and synaptic transmission. In addition to the full-length isoform (TrkB-FL) encoding the active receptor, alternative splicing yields receptors that lack the kinase domain, TrkB-T1, TrkB-T2, and TrkB-T-Shc, suggested as involved in TrkB-FL modulation. Particularly, TrkB-T1 opposes TrkB-FL function via competition for BDNF binding or the formation of inactive heterodimers. Additionally, TrkB-FL-independent signaling has been also suggested for TrkB-T1.

We were interested in the identification of additional neuronal survival pathways downregulated in stroke and sought to characterize their contribution to excitotoxicity. Therefore, we analyzed TrkB expression in models of ischemia and excitotoxicity, and characterized mechanisms acting at the mRNA and protein levels yielding TrkB-FL downregulation and TrkB-T1 upregulation. We investigated the contribution of this imbalance to excitotoxicity and ischemia, and demonstrated a strong association with neurodegeneration. Analysis of human necropsies suggested that TrkB levels might be similarly altered by stroke. These results identified the TrkB isoforms as new therapeutic targets for neuroprotection against excitotoxicity.

Results

Opposite regulation of TrkB isoforms in a model of ischemia. To identify the neuronal survival pathways downregulated in ischemia, we analyzed the TrkB expression and neurodegeneration in a rat model of focal cerebral ischemia, induced by a 1-h middle cerebral artery occlusion (MCAO), followed by reperfusion for 6 h. These conditions produced a moderate decrease in Nissl staining in this artery territory and allowed TrkB characterization in neurons committed to die, whereas avoiding massive death starting after 24 h of reperfusion. Immunohistochemistry with isoform-specific antibodies revealed high TrkB-T1 levels in NeuN-labeled neurons in the ischemic region compared with very low expression in equivalent regions of contralateral neocortex (Figure 1A). Analysis of glial fibrillary acidic protein (Supplementary Figures S2A and B) demonstrated Figure 1. Transient cerebral ischemia induces the opposite regulation of TrkB-FL and T1 isoforms in rat neurons, and TrkB-T1 upregulation is associated with neurodegeneration. Double immunohistochemistry with isoform-specific antibodies of coronal sections of animals subjected to MCAO followed by blood reperfusion (6 h), a model in which damage is limited to the cortex, revealed that neurons in the ischemic region present a large increase in TrkB-T1 levels compared with those in the contralateral hemisphere (A) and an important decrease of TrkB-FL expression (B). Combined immunostaining of TrkB-T1 and NeuN with Fluoro-Jade B (FJB) labeling demonstrated a dramatic association of TrkB-T1 upregulation in the ischemic region with early neuronal degeneration (arrows) (C). However, there was a small number of neurons in this area which did not express TrkB-T1 or stain with FJB (arrowheads). Representative confocal microscopy images corresponding to single sections are shown. The scale bars represent 10 μm.

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scarce astrocytic staining in the ischemic area at this early time and no TrkB-T1 upregulation in glial cells. It was important to establish the cells where TrkB-T1 increased, because this isoform is expressed in neurons and glial cells.\(^{28}\) Immunostaining with a TrkB-FL antibody demonstrated a dramatic reduction of the receptor in the ischemic area compared with the high levels found in neurons in the contralateral region (Figure 1B). The presence of neurons in the ischemic region was demonstrated with NeuN-labeling, which confirmed that TrkB-FL decrease was not due to neuronal death. The switch in TrkB expression from mostly TrkB-FL in contralateral regions to TrkB-T1 in ischemic tissue justified the labeling of both areas with an antibody directed to an epitope present in all TrkB isoforms (panTrkB; Supplementary Figures S2C and D). Finally, to investigate a possible association between TrkB-T1 expression and neurodegeneration, we combined immunostaining for TrkB-T1 and NeuN with Fluoro-Jade B-labeling, a compound that specifically binds to degenerating neurons. In the ischemic area, a dramatic association of high levels of TrkB-T1 expression with neurons undergoing degeneration was observed (Figure 1C, arrows).

**Neuronal-specific TrkB regulation is induced by excitotoxicity.** Excitotoxicity is a primary pathological mechanism of neurodegeneration in stroke.\(^1\) We investigated if excitotoxicity might be causing the TrkB-FL/TrkB-T1 imbalance by using a model reproducing *in vitro* the pathological activation of glutamate receptors. Primary cultures of rat cortices grown 14 days *in vitro* (DIV) were incubated with high concentrations (100 \(\mu\)M) of NMDA or glutamate for 2 h, conditions of relatively high-neuronal viability (Supplementary Figure S3). The NMDAR agonists induced a significant decrease in TrkB-FL (145 kDa) and a reciprocal increase in the truncated forms (collectively designated as tTrkB), as shown by panTrkB (Figure 2A). Other neuronal proteins such as the neuronal-specific enolase (NSE) were not modified by treatment, which demonstrated that TrkB regulation is specific and not a consequence of massive cell death. These effects required the specific activation of NMDARs and were blocked by competitive (DL-AP5) or non-competitive (memantine, ketamine) NMDAR antagonists (D).

**Figure 2** Neuronal-specific regulation of TrkB is induced by NMDAR overactivation in a cellular model of excitotoxicity. (A) Opposite regulation of the TrkB isoforms was induced by agonists of glutamate receptors and prevented by specific NMDAR antagonists. Primary cortical cultures (14 DIV) were treated with NMDA (100 \(\mu\)M) or glutamate (100 \(\mu\)M) with their co-agonist glycine (10 \(\mu\)M) for 2 h, with or without the antagonists DL-AP5 (200 \(\mu\)M), memantine (10 \(\mu\)M), or ketamine (500 \(\mu\)M). TrkB-FL and the other truncated isoforms (tTrkB) that are recognized by panTrkB are indicated. (B) Time course of the TrkB-FL decrease and the tTrkB or TrkB-T1 increase in cultures treated with NMDA for the indicated times. (C) Quantitation of TrkB dysregulation. Relative protein levels of TrkB-FL, tTrkB, or TrkB-T1 (mean \pm S.E.M., \(n=4\) experiments) were established after normalization to NSE and comparison with the protein levels in untreated cells, which were arbitrarily assigned a value of 100%. The effect of NMDA was assessed using a Student’s unpaired *t*-test (*\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\)). (D) Double immunofluorescence of cultures stimulated with NMDA for 1 h using panTrkB or neuronal-specific antibodies demonstrated the neuronal specificity of TrkB expression (arrows) and the lack of TrkB staining in glial cells in the culture (arrowheads). Analysis with isoform-specific antibodies was not possible, because they produced very weak immunofluorescent signals. Representative confocal microscopy images corresponding to single sections are shown. The scale bars represent 10 \(\mu\)m.
ketamine) NMDAR-antagonists (Figure 2A). The GluN2B-selective antagonist ifenprodil prevented TrkB regulation similar to DL-AP5 (Supplementary Figures S4A and B), consistent with the coupling of GluN2B subunits of the extrasynaptic NMDARs to excitotoxic pathways. Also supporting the importance of the source of calcium, the increase of intracellular calcium concentration with thapsigargin, an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, or calcium ionophore A23187 could not mimic the NMDA-induced TrkB regulation (Supplementary Figure S4C). Our mixed cultures, grown in minimum essential medium to reproduce the cellular brain environment and containing a 27 ± 4% of astrocytes, required NMDAR overactivation with NMDA concentrations > 10 μM for TrkB regulation (Supplementary Figures SSA, left panel, and SSB). In comparison, neuronal-enriched cultures in neurobasal medium containing a 4 ± 0.4% of astrocytes presented a higher sensitivity for TrkB-FL downregulation (Supplementary Figure SSB) and neuronal death (Supplementary Figure SSC), probably reflecting reduced glutamate uptake.

A kinetic analysis of TrkB regulation demonstrated that 2 h of NMDA treatment significantly reduced the TrkB-FL expression to 72 ± 5% (P = 0.004; Figures 2B and C). Stimulation over 4 and 8 h further decreased TrkB-FL to 45 ± 2% (P = 0.0001) and 33 ± 3% (P = 0.001), respectively. Conversely, we observed a progressive increase in tTrkB levels (Figure 2B, upper panel), which reached values of 219 ± 30% (P = 0.01) and 304 ± 39% (P = 0.006), relative to untreated cultures 1 and 2 h after NMDA stimulation, respectively (Figure 2C). This increase was partially due to the upregulation of TrkB-T1 (95 kDa; Figure 2B, middle panel), which reached values of 196 ± 18% (P = 0.01) 1 h after NMDA stimulation, relative to untreated cultures, and increased more gradually afterwards (Figure 2C). We analyzed the cell specificity of TrkB regulation by immunofluorescence of neuronal markers NeuN and βIII-tubulin, and panTrkB (Figure 2D). Total TrkB was mainly detected in the soma and dendrites of untreated neurons, also labeled with NeuN (Figure 2D, panel a) or βIII-tubulin (Figure 2D, panel c). After NMDA stimulation for 1 h, a condition that induced prevalence of tTrkB over TrkB-FL (Figure 2B), neurons were still labeled by panTrkB (Figure 2D, arrows), a result resembling that obtained in the ischemic brain (Supplementary Figures S2C and D). Morphological changes characteristic of excitotoxicity and typically preceding death, such as dendritic focal swelling and the formation of varicosities, were found in stimulated neurons (Figure 2D, panels b and d).

Mechanisms of TrkB regulation induced by excitotoxicity. Transient ischemia modifies brain patterns of alternative mRNA splicing. Therefore, we examined changes in the relative mRNA levels of TrkB isoforms after excitotoxic stimulation indicative of a modification of mRNA splicing. Quantitative RT-PCR revealed that TrkB-FL transcripts were significantly reduced to 59 ± 5% (P = 0.00001) and 43 ± 12% (P = 0.04), compared with untreated cells after 2 and 3 h of NMDA stimulation, respectively (Figure 3A). Conversely, TrkB-T1 transcripts were increased significantly after treatment and reached values of 146 ± 13% (P = 0.005) by 2 h of stimulation. We concluded that excitotoxicity induced a progressive increase in the ratio of TrkB-T1 to TrkB-FL mRNA.

We considered other possible mechanisms of TrkB regulation, such as endocytic degradation of BDNF/TrkB complexes that might be mediated by BDNF released after excitotoxic stimulation. Compared with NMDA, BDNF treatment (1–4 h) did not extensively modify TrkB levels, though it induced TrkB tyrosine-kinase phosphorylation and activation (Figure 3B). Quantitation confirmed a significant regulation of TrkB isoforms induced by NMDA compared with stable levels after BDNF treatment, with only a small and transient increase of TrkB-T1 at 1 h (Figure 3C). These results suggest that, in the time window analyzed, BDNF-induced mechanisms did not contribute to TrkB regulation by NMDA. As TrkB-interacting proteins (e.g. NMDARs and kinase D-interacting substrate of 220 kDa/ankyrin repeat-rich membrane spanning (Kidins220/ARMs)) are calpain substrates, and this protease is activated by excitotoxicity, we investigated the possible contribution of calpain to TrkB-FL downregulation. The NMDA-induced decrease in TrkB-FL was largely prevented by the calpain inhibitor calpeptin, which had no effect on TrkB-T1 upregulation (Figure 3D). We tested calpain activity and calpeptin efficacy by analyzing the processing of a well-characterized calpain substrate, FL brain spectrin (240 kDa), which produces breakdown products of 150 and 145 kDa. In contrast, lactacystin did not prevent TrkB-FL regulation, although it increased p53 levels (Figure 3D), a proteasome substrate. The contribution of calpain to TrkB-FL downregulation was quantified. Preincubation with calpeptin upon NMDA treatment greatly restored TrkB-FL levels, which were 68 ± 5% of those obtained in cells incubated only with calpeptin, and significantly higher than those in NMDA-treated neurons (39 ± 3%; P = 0.0003, calpeptin + NMDA versus NMDA; Figure 3E). A parallel comparison failed to show an effect of calpain inhibition in TrkB-T1 regulation (212 ± 26% versus 222 ± 56%, respectively). Collectively, these experiments reveal a dual mechanism of TrkB regulation in excitotoxicity; one major component was extensive calpain-dependent processing of TrkB-FL, and the other element was due to regulation of the relative TrkB-FL and TrkB-T1 mRNA levels.

Characterization of TrkB-FL calpain processing. To further characterize the TrkB-FL processing, we generated a dual lentiviral vector that expresses an N-terminally hemagglutinin (HA)-tagged TrkB-FL and GFP (LV-HA-TrkB-FL/GFP) or two fluorescent proteins (LV-DsRed/GFP) in the same neuron (Figure 4A). These genes were under the control of synapsin promoters, which drove their efficient neuro-specific co-expression. Immunostaining of cultures infected with a low multiplicity of LV-HA-TrkB-FL/GFP confirmed expression of the transducible genes. Compared with non-transduced cells, an increase in TrkB was found only in neurons that expressed GFP (Figure 4B). Immunoblot analysis of cultures infected with increasing multiplicities of LV-HA-TrkB-FL/GFP demonstrated augmented total TrkB-FL levels compared with constant levels of endogenous TrkB-FL in LV-DsRed/GFP-infected cells (Figure 4C). Recombinant TrkB-FL receptors were processed after
NMDA treatment and yielded fragment(s) that contained the N-terminal HA-tag with a mobility of ~95 kDa (Figure 4D). Calpeptin prevented HA-TrkB-FL cleavage, demonstrating that the processing of recombinant and endogenous receptors was calpain-dependent.

We also analyzed in vitro processing of TrkB by purified calpain (Figure 4E). Incubation of extracts containing HA-TrkB-FL with calpain revealed that, contrary to TrkB-T1, recombinant and endogenous TrkB-FL were sensitive to cleavage. This result directly established TrkB-FL as a novel calpain substrate and showed that cleavage occurred downstream but nearby the divergence point with TrkB-T1 sequence. This TrkB-FL fragment, together with TrkB-T1, contributed to the increase of tTrkB detected by pan-TrkB in NMDA-treated neurons (Figure 2B). In conclusion, calpain processing generated a truncated TrkB-FL that lacked the tyrosine-kinase domain and was strikingly similar to TrkB-T1.

Upregulation of TrkB-T1 in human stroke. To approach the possibility that TrkB neuronal survival pathways are also downregulated in human ischemia, we analyzed necropsies from acute ischemic stroke patients, where we compared infarcted cerebral cortex to equivalent regions in the contralateral hemispheres. We characterized TrkB-T1 by immunoblot and found very high levels in the ischemic area compared with contralateral tissue, whereas similar levels of other neuronal proteins (e.g. NSE) were found in both the hemispheres (Figure 5A, left panel). Quantitation of these results showed a mean increase of normalized TrkB-T1 levels of 4.8 times in the infarcted tissue versus the contralateral hemisphere (Figure 5A, right panel), which was not significant, probably because of inter-individual variability, differences in time of tissue collection (2.5–7 h post mortem), and small size of the sample analyzed (n = 3). For TrkB-FL, we found heterogeneous levels in the control tissues (Figure 5A, left panel) probably due to different levels of spontaneous post mortem calpain activation taking place early after death in infarcted and, more importantly, not infarcted tissue.35 Accordingly, almost complete cleavage of spectrin was observed in control and ischemic tissues. To reproduce the instability of TrkB-FL in the postmortem tissue, we analyzed the isoform levels in the brain of the rat.
embryos kept at room temperature for different periods after death (2–8 h; Figure 5B). Processing of TrkB-FL was fast and largely parallel to that of spectrin, although the levels of TrkB-T1 were more stable. This isoform could be still detected by 8 h postmortem, a time point when TrkB-FL and spectrin were already processed. These results suggest that, in stroke, TrkB-FL downregulation induced by ischemia can be only observed at the earliest postmortem times (Figure 5A, patient 15), and that TrkB-T1 upregulation is not related to spontaneous calpain activation. Next, we performed immunohistochemistry of human infarcted cortex and confirmed the increase in TrkB-T1 compared with the very low levels found in the contralateral hemisphere (Figure 5C). The identification of post-mitotic neurons with NeuN antibodies revealed that TrkB-T1 upregulation in stroke was mainly neuro-specific (Figure 5C) as demonstrated before in the animal model (Figure 1A and Supplementary Figures S2A and B). The heterogeneity of NeuN levels in the ischemic region was suggestive of neurodegeneration, which may be associated with TrkB-T1 upregulation.

**Recovery of TrkB-FL/TrkB-T1 balance confers neuroprotection against excitotoxicity.** Dysregulation of TrkB isoforms induced by excitotoxicity may contribute to neuronal death and, therefore, reversion of these changes...
would confer neuroprotection. To test this hypothesis, we investigated the consequences in neuronal viability of an increase in TrkB-FL expression. After LV-HA-TrkB-FL/GFP infection, the combined levels of wild-type and recombinant TrkB-FL were higher compared with the LV-DsRed/GFP-infected cultures (Figure 6a), although both the TrkB-FL forms were still processed by NMDA-activated calpain. This increase only partially counteracted one of the mechanisms of TrkB regulation induced by excitotoxicity and had no effect on TrkB-T1 upregulation (Figure 6a) or neuronal viability (Figure 6b). Next, we explored the effect of counteracting the TrkB-T1 increase by using microRNAs (miRNAs). Five double-stranded oligonucleotides (Supplementary Figure S6) encoding pre-miRNAs specific for target sequences in TrkB-T1 mRNA 3′-UTR (Supplementary Figure S7A) were used to produce pcD/miT1 plasmids, which were initially tested in a heterologous system (Supplementary Figure S7B). Plasmids showing the highest efficiency in TrkB-T1 interference...
compared with the pcD/miC control (pcD/miT1(2310) and pcD/miT1(2178)) were chosen for further analysis. To produce neuro-specific TrkB-T1 interference, these pre-miRNAs were cloned under the control of a synapsin promoter to produce lentiviruses LV-miT1(2178)/GFP and LV-miT1(2310)/GFP (herein, LV-miT1/GFP). Transcription of miRNAs was controlled by RNA polymerase II and was cell type-specific. Lentivirus LV-miC/GFP contains negative-control pre-miRNAs sequences. Efficient and specific interference of basal TrkB-T1 expression was obtained by double infection with LV-miT1(2178)/GFP plus LV-miT1(2310)/GFP (m.o.i. = 1 for each virus) or LV-miT1(2310)/GFP with LV-miC/GFP (m.o.i. = 1 for each virus). No changes were observed for TrkB-FL or other analyzed proteins. Double infection with LV-miT1(2178)/GFP and LV-miT1(2310)/GFP strongly interfered with the TrkB-T1 upregulation that was induced by NMDA compared with cultures infected with LV-miC/GFP (m.o.i. = 2). Relative neuronal viability (mean ± S.E.M., n = 6) of cells infected with LV-miT1(2178)/GFP and LV-miT1(2310)/GFP (m.o.i. = 1 for each virus), or LV-miC/GFP (m.o.i. = 2), treated or not treated with NMDA. Student’s unpaired t-test revealed no significant differences in viability between cells infected with these viruses compared with the PC/miC control. We reasoned that it might be necessary to simultaneously counteract both the mechanisms of TrkB dysregulation to protect neurons from excitotoxic damage. Therefore, we generated another lentivirus that produced TrkB-FL and the two more efficient pre-miRNAs (LV-HA-TrkB-FL/ 2xmiT1) in the same neuron (Figure 7a). This virus strongly interfered with basal TrkB-T1 expression and increased TrkB-FL levels compared with untreated LV-DsRed/GFP-infected cells (Figure 7b). Moreover, after NMDA stimulation, LV-HA-TrkB-FL/2xmiT1 prevented TrkB-T1 upregulation and induced higher TrkB-FL levels compared with LV-DsRed/GFP-infected cultures (Figure 7b). We compared the effect of double counteraction of TrkB dysregulation on neuronal viability with respect to single strategies. The viability of untreated cultures infected with lentiviruses LV-DsRed/GFP, LV-HA-TrkB-FL/2xmiT1, LV-HA-TrkB-FL/GFP, LV-miC/GFP, or LV-miT1/GFP was very similar (Supplementary Figure S8), suggesting that an increase in TrkB-FL and/or a decrease in TrkB-T1 did not enhance basal survival pathways. In contrast, infected neurons responded differently to 2 h of NMDA compared with the PC/miC control (pcD/miT1(2310) and pcD/miT1(2178)) were chosen for further analysis. To produce neuro-specific TrkB-T1 interference, these pre-miRNAs were cloned under the control of a synapsin promoter to produce lentiviruses LV-miT1(2178)/GFP and LV-miT1(2310)/GFP (Figure 6c). Strong interference of basal TrkB-T1 expression was produced by LV-miT1(2310)/GFP-infection, which was further increased by mixed infection with LV-miT1(2178)/GFP, a virus having only moderate effects by itself (Figure 6d). Interference was specific for TrkB-T1 and not observed for TrkB-FL or other proteins. Additionally, the combined infection with LV-miT1/GFP viruses strongly prevented the TrkB-T1 upregulation induced by excitotoxicity in LV-miC/GFP-infected cells (Figure 6e). However, it had no effect on TrkB-FL downregulation (Figure 6e) or neuronal viability (Figure 6f).

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Neuronal viability was reduced by NMDA to 75 ± 1% in LV-HA-TrkB-FL/2xmiT1-infected cells, a value that was significantly higher than that obtained in cultures infected with LV-DsRed/GFP (52 ± 3%, P = 0.0001) or the other viruses (Figure 7c). A time-course analysis in LV-HA-TrkB-FL/2xmiT1-infected neurons demonstrated that the greatest differences in the kinetics of neuronal death were obtained during the initial period of excitotoxicity, though neuronal viability was higher at the various times tested, compared with LV-DsRed/GFP-infected cells (Figure 7d). After 4 h of NMDA stimulation, the viability of LV-HA-TrkB-FL/2xmiT1-infected cultures was 48 ± 3%, a value significantly higher than that found in the LV-DsRed/GFP-infected cultures (27 ± 2%, P = 0.0005). As protection was observed in LV-HA-TrkB-FL/2xmiT1-infected neurons, we analyzed TrkB activity finding high levels of phosphorylated TrkB-FL in untreated cultures that decreased progressively over the time of NMDA treatment similar to total TrkB-FL (Figure 7b). Therefore, these results demonstrated that the dysregulation of normal TrkB-FL/TrkB-T1 balance is an important mechanism of neuronal death induced by excitotoxicity, and the simultaneous increase in TrkB-FL expression and interference of TrkB-T1 synthesis has a neuroprotective effect against these insults.

**Discussion**

Excessive activation of NMDARs is central to degeneration and death of the adult neurons in ischemia and other disorders by mechanisms that are not completely understood. Here, we unveiled a TrkB-FL/TrkB-T1 imbalance as one neurodegeneration mechanism in ischemia. A dysregulation of TrkB was observed in neurons of infarcted human brain and rat neurons that were subjected to in vivo or in vitro excitotoxicity. One of the mechanisms contributing to the TrkB regulation altered the physiological ratio of mRNAs encoding for TrkB isoforms and favored the expression of TrkB-FL in untreated cultures that decreased progressively over the time of NMDA treatment similar to total TrkB-FL (Figure 7b). Therefore, these results demonstrated that the dysregulation of normal TrkB-FL/TrkB-T1 balance is an important mechanism of neuronal death induced by excitotoxicity, and the simultaneous increase in TrkB-FL expression and interference of TrkB-T1 synthesis has a neuroprotective effect against these insults.
stimulation\textsuperscript{2} may decrease TrkB transcription and shift the splicing of TrkB precursor mRNA. However, we will need to consider other possible mechanisms induced by excitotoxicity. The second and major mechanism of TrkB regulation was calpain processing of TrkB-FL, unveiled here as a new calpain substrate. A detailed analysis of TrkB-FL truncation in stroke patients was hampered by postmortem spontaneous-calpain activation in non-infarcted tissue.\textsuperscript{36} However, in a cellular model, we were able to establish that calpain activation by excitotoxicity generated a truncated TrkB-FL receptor similar to TrkB-T1, which may act as an additional dominant-negative protein. All together, the decrease of BDNF/TrkB signaling will impact neuronal survival and brain function.

Ischemia also regulates other proteins critical to neuronal survival related to TrkB. The scaffolding protein PSD-95, which is central to NMDAR regulation, is cleaved by calpain, survival related to TrkB. The scaffolding protein PSD-95, negative protein. All together, the decrease of BDNF/TrkB signaling will impact neuronal survival and brain function.

Materials and Methods

**Plasmids.** Plasmid pRC-CMV-HA-TrkB-FL, provided by Y Barde (Biozentrum, Basel, Switzerland), encodes rat TrkB-FL with a HA epitope inserted at the N-terminus. Oligonucleotides 5'GGGAGGCGGTAGTGGAGGACACGGGCGTCTGTGCGGCGGCGCTGGTACGTAGGAGG AGGACACGGGCGTCGGCGGACAGC 3' and 5'GACAAAAGGAGGCAGGCGCCGCGGTTGAGGAGCGTCGTGACGGGCGCTGGTACGTAGGAGG AGGACACGGGCGTCGGCGGACAGC 3' were used to introduce BamHI and NotI sites flanking this cDNA. Nucleotides mutated in the TrkB-FL cDNA are in bold, and restriction sequences are underlined. This BamHI-NotI cDNA fragment was sub-cloned, substituting DsRed in LV-DsRed/GFP\textsuperscript{-}HI–GFP\textsuperscript{-}I cDNA fragment was sub-cloned, substituting DsRed in LV-DsRed/GFP\textsuperscript{-}HI–GFP\textsuperscript{-}I sites flanking this cDNA. Nucleotides mutated in the TrkB-FL cDNA are in bold, and restriction sequences are underlined. 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(infarction) and contralateral hemispheres was collected within the first 7 h after death, snap-frozen in liquid nitrogen, and stored at −80 °C for immunoblotting or fixed with formalin for immunohistochemistry.

**Immunoblot analysis.** For human brain extracts, tissue was homogenized in lysis buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM CaCl₂, 0.05% Brij-35 (vol/vol), 0.02% NaN₃ (vol/vol), and 1% Triton X-100 (vol/vol) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM aprotinin). Cortical cultures were lysed in RIPA buffer as described previously.13 and equal amounts of protein (25–50 μg) were analyzed by immunoblotting, using the primary and secondary antibodies indicated in the Supplementary Information. Immuno-complexes were detected with a Bioluminescence kit from Perkin-Elmer (Boston, MA, USA). Densitometric analysis was performed with NIH Image analysis software. The results were normalized to levels of NSE in the same samples and are represented as relative values in reference to the values obtained in the untreated cells.

**Immunofluorescence.** The immunofluorescence procedure was described previously.13 Confocal images were acquired using a Radiance 2000 confocal (BioRad, Hercules, CA, USA) coupled to an inverted Axiovert S100 TV microscope (Zeiss, Oberkochen, Germany) with a 63x Plan-Apochromat oil immersion objective. Immunohistochemistry. Frozen sections (12 μm) of human specimens were prepared using a cryostat, post-fixed in ice-cold acetone for 15 min, and washed in 0.05% Tween (vol/vol)-PBS. Sections were blocked with 10% BSA (wt/vol) (wt/vol) Triton X-100 (vol/vol) in PBS for 1 h at room temperature, and incubated overnight at 4 °C with primary antibodies. After incubation with secondary antibodies, sections were mounted on coverslips using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) or stained with Fluoro-Jade B (Chemicon, Temacula, CA, USA) according to manufacturer's instructions. Confocal images were acquired as described for immunofluorescence.

**Calpain in vitro proteolysis.** Protein extracts were prepared using RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM EDTA, and 1 mM EGTA. Calpain I (80 U/ml) was added to diluted amounts of protein (25–50 μg) and incubated for 30 min at 37°C. The reaction was terminated with 10 mM phenylmethylsulfonyl fluoride and 7 μg/ml aprotinin. Cortical cultures were lysed in RIPA buffer as described previously.13 and equal amounts of protein (25–50 μg) were analyzed by immunoblotting, using the primary and secondary antibodies indicated in the Supplementary Information. Immuno-complexes were detected with a Bioluminescence kit from Perkin-Elmer (Boston, MA, USA). Densitometric analysis was performed with NIH Image analysis software. The results were normalized to levels of NSE in the same samples and are represented as relative values in reference to the values obtained in the untreated cells.

**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)