Prevention of excitotoxicity-induced processing of BDNF receptor TrkB-FL leads to stroke neuroprotection

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Abstract

Neuroprotective strategies aimed to pharmacologically treat stroke, a prominent cause of death, disability, and dementia, have remained elusive. A promising approach is restriction of excitotoxic neuronal death in the infarct penumbra through enhancement of survival pathways initiated by brain-derived neurotrophic factor (BDNF). However, boosting of neurotrophic signaling after ischemia is challenged by downregulation of BDNF high-affinity receptor, full-length tropomyosin-related kinase B (TrkB-FL), due to calpain-degradation, and, secondarily, regulated intramembrane proteolysis. Here, we have designed a blood–brain barrier (BBB) permeable peptide containing TrkB-FL sequences (TFL457) which prevents receptor disappearance from the neuronal surface, early induced after excitotoxicity. In this way, TFL457 interferes TrkB-FL cleavage by both proteolytic systems and increases neuronal viability via a PLCγ-dependent mechanism. By preserving downstream CREB and MEF2 promoter activities, TFL457 initiates a feedback mechanism favoring increased levels in excitotoxic neurons of critical prosurvival mRNAs and proteins. This neuroprotective peptide could be highly relevant for stroke therapy since, in a mouse ischemia model, it counteracts TrkB-FL downregulation in the infarcted brain, efficiently decreases infarct size, and improves neurological outcome.

Keywords cell-penetrating peptides; excitotoxicity; neuroprotection; stroke; TrkB

Subject Categories Neuroscience; Pharmacology & Drug Discovery; Vascular Biology & Angiogenesis

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Introduction

Stroke is a leading cause of death, disability, and dementia. In the ischemic type, treatments are largely limited to mechanical strategies or pharmacological therapy with thrombolytic drugs. The decrease in brain perfusion due to vessel occlusion results in formation of the infarct core, irreversibly damaged tissue surrounded by an area of penumbra that is functionally impaired but metabolically active. However, infarct frequently expands to this area due to secondary neuronal death caused by overstimulation of the N-methyl-D-aspartate type of glutamate receptors (NMDARs) and consequent excitotoxicity. This damage is also associated with other acute and chronic CNS disorders (Choi, 1988), adding relevance to the development of excitotoxicity therapies. A promising approach is enhancement of survival pathways such as those regulated by brain-derived neurotrophic factor (BDNF) through binding to tropomyosin-related kinase B (TrkB) receptors. Remarkably, BDNF/TrkB potentiation would be protective in acute stroke and stimulate neurorepair at later stages (Berretta et al., 2014).

Promotion of neuronal survival by BDNF is mediated by dimerization, increased tyrosine kinase (TK) activity, and transphosphorylation of its high-affinity receptor full-length TrkB (TrkB-FL). Activation triggers three interconnected cascades: MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase), PI3K/Akt (phosphatidylinositol 3-kinase/v-Akt murine thymoma viral oncogene homolog), and PLCγ (phospholipase Cγ; Huang & Reichardt, 2003; Reichardt, 2006). Among other functions, these cascades activate prosurvival transcription factors (TFs) CREB (cAMP response-element binding protein; Bonni et al., 1999) and MEF2 (myocyte enhancer factor 2; Liu et al., 2003) that regulate the expression of target genes, including those coding BDNF (Tao et al., 1998; Lyons et al., 2012), TrkB (Deogracias et al., 2004) or NMDAR subunits (Kraicz et al., 1998; Desai et al., 2002; Lau et al., 2004). Neurons also express TrkB-T1, a truncated isoform lacking the TK and having a short cytoplasmic domain
with an isoform-specific sequence. It is considered a dominant-negative receptor that inhibits BDNF signaling by forming heterodimers with TrkB-FL, and sequestering and translocating BDNF (Haapasalo et al., 2001). Upon binding, both isoforms are rapidly and efficiently internalized in a clathrin-dependent way (Zheng et al., 2008; Huang et al., 2009) and form signaling endosomes (Huang et al., 2009). While TrkB-T1 predominantly recycles back to cell surface by a default mechanism, TrkB-FL recycling is less efficient, relies on TK activity, and is regulated by binding of Hrs (hepatocyte growth-factor-regulated tyrosine kinase substrate) to a juxtamembrane region between the transmembrane (TM) and TK domains (Huang et al., 2009).

BDNF/TrkB signaling becomes profoundly aberrant in stroke (Berretta et al., 2014; Tejeda & Diaz-Guerra, 2017). A permanent BDNF decrease is found in the infarct core, while a long-lasting upregulation, proposed to be a neuroprotective mechanism, takes place in the penumbra (Ferrer et al., 2001; Madinier et al., 2013). However, other studies rejected BDNF involvement in post-stroke recovery (Hirata et al., 2011), a likely explanation being pathological downregulation of BDNF receptors. Three independent mechanisms induced by excitotoxicity act on TrkB: (i) An inversion of mRNA ratios disfavors TrkB-FL expression over TrkB-T1 (Gomes et al., 2012; Vidaurre et al., 2012); (ii) TrkB-FL cleavage by Ca\(^{2+}\)-dependent calpain generates a truncated receptor similar to TrkB-T1 (Gomes et al., 2012; Vidaurre et al., 2012); and (iii) regulated intramembrane proteolysis (RIP) of both isoforms by metalloproteinase/\(\gamma\)-secretase action sheds identical ectodomains acting as BDNF scavengers (Tejeda et al., 2016). While RIP highly contributes to TrkB-T1 downregulation in ischemia, it is only a secondary mechanism for TrkB-FL, mainly processed by calpain. The importance of calpain for TrkB-FL dysregulation in excitotoxicity-associated disorders other than stroke has been confirmed in epilepsy (Danelon et al., 2016) and Alzheimer disease (AD; Jeronimo-Santos et al., 2015). Calpain activation (Adamec et al., 2002) and TrkB-FL loss (Allen et al., 1999; Ferrer et al., 1999) had been observed before in the brain of AD patients, while a TrkB-FL calpain processing site was mapped by Edman sequencing in neurons treated with A\(\beta\) (Jeronimo-Santos et al., 2015). Altogether, the mechanisms described impair BDNF signaling and point to TrkB as a therapeutic target for neuroprotection in disorders associated with excitotoxicity.

To preserve BDNF-regulated survival pathways, we have designed and characterized cell-penetrating peptides (CPPs) based on the BDNF receptor. They contain a Tat sequence, which allows attached cargoes to cross the blood–brain barrier (BBB) and plasma membrane (Regberg et al., 2013), and different TrkB-FL sequences that we hypothesized might control receptor stability and function in excitotoxicity. One peptide (TFL457) proved to interfere TrkB-FL calpain processing and RIP in rat primary neurons subjected to in vitro excitotoxicity. The primary mechanism of TFL457 action is to maintain TrkB-FL in the cell surface, apart from the proteolytic machinery activated in excitotoxicity. The preserved receptor triggers a feedback survival mechanism mediated by PLC\(\gamma\), and CREB and MEF2 promoter activities. Importantly, this neuroprotective peptide also counteracts TrkB-FL downregulation in mouse ischemia, where it efficiently decreases infarct size and improves neurological outcome, unraveling a highly relevant strategy for stroke therapy.

### Results

**TrkB-FL juxtamembrane region is a rational target for the design of CPPs interfering receptor downregulation**

To approach the design of CPPs able to preserve TrkB-FL levels in excitotoxicity and ischemia, we focused in the receptor inter-domain sequence located between the TM and proximal TK domains (aa 453–536) for several reasons. Firstly, a calpain cleavage site had been mapped between residues N520/S521 in this region in an AD model (Fig 1A, red arrow; Jeronimo-Santos et al., 2015). Calpains often proteolyze a limited number of specific sites inside substrates inter-domains and, thus, additional processing sites in TrkB-FL could not be excluded. Secondly, sequences in the juxtamembrane region have been shown to be important for the regulation of different aspects of TrkB-FL location and function via protein interaction (Huang et al., 2009, 2011; Guo et al., 2017; Zamani et al., 2018). Lastly, we found an intricately disordered region (IDR) inner to this TrkB-FL juxtamembrane sequence (Fig EV1; Kozlowski & Bujnicki, 2012). IDRs are considered central units of protein function and regulation due to their ability to establish multiple interactions (Dunker et al., 2008) and their potential to act as weak signals for degradation (Tompa et al., 2008). Interestingly, in silico analysis of this TrkB-FL region using several predictive algorithms for calpain processing [CAMPDB (DuVerle et al., 2011), GPS-Calpain Cleavage Detector (Liu et al., 2011) and SitePrediction (Verspurten et al., 2009)] showed that predictions made by at least two algorithms mostly clustered in two areas (Fig 1A, black arrows) inside the described IDR (Fig EV1). Three of them were found nearby the TM in residues shared by all isoforms (light blue) and of potential functional importance according to a heatmap representation of all possible mutations (Hecht et al., 2015). We also found a second cluster of calpain sites in this same IDR and two more predictions in the proximal TK region.

Next, we selected four sequences potentially important for TrkB-FL regulation (Fig 1A, black rectangles) and generated CPPs containing them fused to a HIV-1 Tat basic domain, which confers membrane permeability and the capability of crossing the BBB to attached cargoes (Regberg et al., 2013; Fig 1B). These CPPs contained, respectively, TrkB-FL aa 457–471 (TFL457), 482–495 (TFL482), 518–531 (TFL518), and 541–555 (TFL541). As a negative control, we designed a similar Tat peptide with unrelated sequences corresponding to c-Myc (Fig 1B, TMyc). We verified TMyc capability to cross the membrane by incubating primary cortical cultures with biotin-labeled TMyc (Bio-TMyc; Fig 1C, panel b). Compared to untreated cultures (panel a), Bio-TMyc was detected in the cell body and neurites of the majority of neurons present in the culture, identified by NeuN (panel b, arrowheads). Quantitation of peptide entry showed that 83 ± 4% (n = 5) of neurons had internalized it (Fig EV2). TMyc suitability as a control peptide was also tested in neuronal viability assays. Treatment with NMDAR co-agonists, NMDA (100 \(\mu\)M) and glycine (10 \(\mu\)M; herein denoted NMDA), dramatically decreased neuronal viability which was not statistically modified by peptide preincubation, both in basal or excitotoxic conditions (Fig 1D). This result shows that Tat-based CPPs have no generic effects on neuronal viability and can be used to test potential neuroprotective sequences.
TFL₄₅₇ interferes TrkB-FL downregulation and prevents acute and chronic excitotoxicity

Once validated the feasibility of our CPP approach, we analyzed the ability of the designed peptides to prevent TrkB-FL processing in excitotoxicity. Cultures were incubated with peptides TMyc, TFL₄₅₇, TFL₄₈₂, or TFL₅₄₁ before chronic NMDA treatment. Analysis of TrkB levels with an antibody recognizing all isoforms (panTrkB) showed that NMDA induced a significant decrease in TrkB-FL (145 kDa) and a reciprocal increase in truncated forms (TrkB-T₁ and calpain-truncated TrkB-FL, collectively named tTrkB; Fig 2A), as described (Vidaurre et al., 2012). In cultures pretreated with TMyc, TFL₄₈₂, or TFL₅₄₁, a similar TrkB-FL reduction was observed. Only TFL₄₅₇ interfered TrkB-FL downregulation (Fig 2B): Normalized receptor levels reached a value of 80 ± 7% compared with the drastic reduction observed in control cultures (40 ± 4%) or those treated with other CPPs. Calpain activation in excitotoxicity was confirmed by accumulation of characteristic spectrin breakdown products (BDPs; 150 and 145 kDa). Neuron-specific enolase (NSE), not affected by NMDA, was used as a loading control. Next, we established the effect of these peptides on neuronal viability (Fig 2C). No significant differences were found in cultures preincubated with TFL₄₈₂ or TFL₅₄₁ compared with cells treated with TMyc, which showed a marked time-dependent decrease in neuronal viability. In contrast,
TFL457 increased neuronal viability at all evaluated times. Thus, in cultures preincubated with TFL457, viability reached values of 50 ± 5% at 2 h of NMDA treatment, significantly higher than those obtained with TMyc (17 ± 2%). Likewise, we explored peptide TFL457 and found that it was not able to maintain TrkB-FL levels and was not neuroprotective (Fig 2D and E). Altogether, we find a very good correlation between interference of TrkB-FL processing and neuronal death prevention, actions exerted by TFL457 but absent for the other peptides.

Finally, we investigated TFL457 neuroprotective effect in a different experimental paradigm where acutely induced damage precedes treatment. After brief excitotoxic stimulation, cells were treated with the NMDAR antagonist DL-AP5, together with TMyc or TFL457, and neuronal viability was established 20 h later (Fig 2F). Excitotoxicity is an irreversible process that, after a critical period of time, cannot be blocked or reverted by NMDAR antagonists (Hartley & Choi, 1989). Hence, neuronal viability was reduced to 37 ± 9% by acute excitotoxicity in the presence of TMyc. However, damage was significantly lower in neurons treated with TFL457 (71 ± 10), proving that TFL457 is effective on neurodegenerative processes downstream NMDAR overactivation. Altogether, above results demonstrate that TFL457 is neuroprotective either when present before a chronic excitotoxic stimulus or administered after acute damage.

**TrkB-FL preserved by TFL457 action maintains Y816 phosphorylation and PLCγ-dependent pathways required for neuroprotection**

BDNF binding induces transphosphorylation of TrkB-FL residues Y515 and Y816, outside the TK region, acting as anchor sites for adaptor proteins (Reichardt, 2006; Fig 3A). We verified the increase in TrkB phosphorylation induced by BDNF (1 h) with phosphospecific antibodies (pY515 and pY816; Appendix Fig S1A, upper panels). Notably, in our cellular model of excitotoxicity, pretreatment with BDNF for 1 h could not prevent the decrease in neuronal viability (Appendix Fig S1B) or interfere the processing of TrkB-FL (Appendix Fig S1C) induced by NMDA. Basal TrkB activation, observed in this experiment (Appendix Fig S1A, lower panels) as before (Du et al, 2003; Gomes et al, 2012; Vidaurre et al, 2012), might be due to spontaneous neuronal activity of dissociated cultures (Pasquale et al, 2017) and mediated by calcium-influx through NMDARs and calcium channels (Du et al, 2003). Next, we were interested in establishing the phosphorylation status of the TrkB-FL receptor preserved in excitotoxic conditions by TFL457 action. Compared to basal levels of TrkB phosphorylation in the absence of NMDA, an important decline was induced by the NMDAR agonist in TMyc presence (Fig 3B and C). In contrast, a more sustained Y816 phosphorylation was observed with TFL457, both after direct lysate analysis (78 ± 9% vs. 46 ± 9) or following immunoprecipitation with a phosphoTyr-specific antibody (Fig 3D). No significant TFL457 effect was found in pY515 levels (Fig 3C), despite TrkB-FL preservation.

To complete characterization of the downstream signaling pathways involved in TFL457 neuroprotection, cultures were first preincubated with selective inhibitors of PI3K, MAPK/ERK, or PLCγ activities (Fig 3A), followed by peptide and NMDA treatment. Neuronal viability of cultures expressed to Wortmannin or U0126 and then treated with TFL457 was similar to cells with no inhibitor (Fig 3E). Only PLCγ inhibition with U-73122 blocked TFL457 effects, viability reaching values significantly lower than those obtained in cultures pretreated with TFL457 but no inhibitor (27 ± 5% vs. 45 ± 4%) and similar to those found with TMyc and U-73122. Thus, the receptor preserved in excitotoxicity by TFL457 action maintains Y816 phosphorylation and PLCγ-dependent signaling which is required for the neuroprotective effects. In contrast, pathways associated with Y515 phosphorylation are not involved in TFL457 neuroprotection.

**TFL457 preserves additional survival proteins downstream TrkB signaling in excitotoxicity**

PLCγ activation increases the activity of Ca2+-dependent pathways (Numakawa et al, 2001) which, among other effects, activate CREB. Thus, we investigated CREB activity in cultures preincubated with different concentrations of TMyc or TFL457 (Fig 4A). Levels of total CREB or S133 phosphorylated CREB (pCREB), generally considered the active protein, showed a decrease induced by NMDA in the presence of TMyc. Reduction of pCREB is likely due to phosphatase activation, which causes CREB shut-off, blockade of BDNF expression, and death of mature neurons in excitotoxicity (Hardingham et al, 2002). Calpain processing might also contribute to CREB decrease, as suggested by CREB truncation in AD brains (Jin et al, 2013). Interestingly, levels of CREB and pCREB were preserved by TFL457.
Figure 2.
in a dose-dependent way, parallel to peptide effects on neuronal viability (Appendix Fig S2).

We further characterized TFL457 effects on survival pathways by time-course experiments (Fig 4B and C). As expected, a restrained TrkB-FL reduction was found at 2 and 4 h of NMDA treatment in the presence of TFL457 compared with TMyc. The effect was isoform-specific, and TFL457 did not prevent the gradual increase in TrkB-FL characteristic of in vitro excitotoxicity (Vidaurre et al., 2012). Next, we analyzed TFL457 effects on prosurvival CREB and MEF2D proteins. The latter belongs to a family of TFs involved in neuronal survival promotion downstream BDNF/TrkB activation (Liu et al., 2003) or synaptic activity (Linseman et al., 2003). Similarly to CREB, MEF2 targets include Bdnf and, thus, there might be a feedback mechanism between BDNF expression and TF activation. A severe decrease in MEF2D was found in cultures subjected to excitotoxicity in TMyc presence, probably due to action of caspases or calpain (Tang et al., 2005; Wei et al., 2012), while this TF was better preserved by TFL457 (Fig 4C). As before, TFL457 had important effects in CREB and pCREB levels.

The intricate interplay of glutamate and neurotrophin-signaling made us wonder if TFL457 might also maintain NMDAR-dependent survival pathways. Excitatory neurotransmission stimulates BDNF synthesis (Hardingham et al., 2002) which modulates NMDAR activity through TrkB activation (Bamji et al., 2006). Additionally, both signaling cascades activate CREB and MEF2. We analyzed NMDAR obligatory subunit GluN1 and GluN2A, predominantly confined to synapses of mature neurons and mostly related to survival (Papadia & Hardingham, 2007). As expected, NMDA induced subunit downregulation, TFL457 is not only able to partly preserve BDNF/TrkB signaling but also interconnected NMDAR survival pathways that cooperate to sustain CREB and MEF2D levels. These prosurvival TFs might then regulate expression of members of both cascades and, thus, amplify TFL457 effects on survival.

Promoter activity and mRNA levels of CREB and MEF2-regulated genes are preserved by TFL457 in excitotoxicity

First, we explored if CREB and MEF2 promoter activities were maintained in excitotoxicity by TFL457 action. Transcription dependent on these TFs is key in neuronal survival induced by neurotrophins
We performed reporter assays using promoters with minimal MEF2 or CREB responsive elements (respectively, pMEF2 or pCRE; Woronicz et al., 1995; Deogracias et al., 2004) controlling luciferase expression. In pMEF2-transfected cells (Fig 5A), luciferase activity was 90% after NMDA treatment in the presence of TFL457, a value significantly higher than that obtained with TMyc (58%). This effect was specific and lost by mutation of MEF2 responsive elements. In pCRE-transfected cells (Fig 5B), excitotoxicity decreased luciferase activity to 36% and 64%, respectively, in TMyc or TFL457-treated cells, proving a TFL457 effect also on CREB-dependent regulation. Treatment with KG-501, a compound that disrupts interactions of CREB-binding protein (CBP) with different TFs that include CREB, decreased all luciferase values that now were similar in excitotoxic cultures treated with TFL457 or TMyc. Similar reporter assays were performed with promoter regions corresponding to CREB and/or MEF2-regulated genes expressing prosurvival proteins GluN1 (Bai et al., 2003) and GluN2A (Desai et al., 2002), BDNF (Tao et al., 1998), and TrkB (Deogracias et al., 2004). The decrease in GluN1 and GluN2A promoter activities induced by excitotoxicity was again counteracted by preincubation with TFL457 compared with TMyc (Fig 5C). For BDNF and TrkB, a significant TFL457 effect could not be observed with the severe excitotoxic conditions used above, but a strong effect was unveiled under milder excitotoxicity (Fig 5D).
Figure 5. Promoter activity and mRNA levels of CREB and MEF2-regulated genes are preserved by TFL457 in excitotoxicity.

A Effect of TFL457 on MEF2-promoter activity. Cultures transfected with pMEF2 (two minimal wild-type MEF2 elements) or pMEF2mut (mutant) were preincubated with CPPs (25 μM, 30 min) and treated with NMDA (100 μM, 2 h). Means ± SEM of luciferase activities obtained in excitotoxicity, relative to values found in cells treated with same peptide and no NMDA, are presented. Significance was analyzed by Student’s t-test (*P = 0.039, n.s. = non-significant, P = 0.79; n = 8).

B Effect on CRE-promoter activity. pCRE contains two minimal CREs. Peptide preincubation was as above with or without KG-501 (10 μM). Mean ± SEM luciferase activities is given relative to values in cells treated with the same peptide but no NMDA or KG-501. Differences found in excitotoxicity were analyzed by ANOVA test followed by post hoc Tukey’s HSD test (*P = 0.023; **P = 0.003; n.s. = non-significant, P = 0.20; n = 6).

C Effect on NMDAR-subunit promoters. Cells transfected with pGluN1 or pGluN2A were treated and analyzed as in panel (A). Significance was analyzed by Student’s t-test (pGluN1, *P = 0.016; pGluN2A, *P = 0.029; n = 14).

D Effects on BDNF promoter III or TrkB promoter. Cells transfected with pIIIBDNF or pTrkB were processed as above but using 20 μM NMDA for 4 h. Data are presented and analyzed as in panel (A). Significance was analyzed by Student’s t-test (pCRE, **P = 0.0098; pIIIBDNF, *P = 0.029; pTrkB, *P = 0.033).

E Effects of TFL457 on mRNA levels of CREB/MEF2-regulated genes. Total RNA was extracted from cultures preincubated with CPPs (25 μM, 30 min) and treated or not with NMDA (100 μM, 4 h). Levels of mRNA were normalized to NSE (genes expressed in neurons, left panel) or GAPDH (neuronal and glial expression, right panel). Means ± SEM of levels obtained in excitotoxicity relative to values found in cells treated with same peptide and no NMDA are presented. Differences found in excitotoxicity were analyzed by ANOVA test followed by post hoc Tukey’s HSD test (GluN1, ***P = 0.00096; GluN2A, P = 0.98; TrkB-FL, ***P = 0.00037; BDNF, *P = 0.045, n = 8).
These results in promoter activities suggested that, for genes regulated by these TFs, TFL457 might be able to tune transcriptional changes induced by excitotoxicity and favor neuroprotection. In accordance with previous results (Gascon et al., 2005), we observed a decrease in levels of Girt1 mRNA, encoding GluN1, in cells treated with TMyc and NMDA (Fig 5E). This decay was reverted by TFL457 action. For Ntrk2, we could also verify the decrease in the mRNA encoding the TrkB-FL isoform induced by NMDA in the presence of TMyc (Vidaurre et al., 2012), changes again counteracted by TFL457. A similar tendency was found for GluN2A regulation. Finally, BDNF mRNA increased in excitotoxic conditions in the presence of TMyc according to published results (Zafra et al., 1991), accumulation exacerbated by TFL457. In conclusion, TFL457 not only preserves CREB and MEF2 proteins in excitotoxicity but also their regulatory activities which very likely modify transcription of target genes central to neuronal survival, contributing in this way to neuroprotection.

Preservation of TrkB-FL in the cell surface by TFL457 precedes interference of RIP and calpain processing in excitotoxicity

We investigated next the primary mechanism of TFL457 action on TrkB-FL stability. Receptor processing by calpain or RIP produces characteristic intracellular fragments that can be recognized by C-ter antibodies (TrkB-FL Ct; Tejeda et al., 2016). Remarkably, interference of TrkB-FL cleavage by TFL457 in excitotoxicity was associated with a significant decrease in both RIP and calpain C-ter fragments, respectively, f42/39 (42–39 kDa) and f32 (32 kDa; Fig 6A and B). We hypothesized that TFL457 might be interfering an excitotoxicity-induced process upstream of TrkB-FL processing and neuronal death. Therefore, the combination of TFL457 with specific calpain (Fig 6C) or metalloproteinase (Fig 6D) inhibitors would not have additive neuroprotective effects. For TMyc-treated cultures, we found a modest but significant effect of calpain inhibitors on neuronal viability (31 ± 2% vs. 19 ± 2%), according to previous observations (Rami et al., 1997; Gerencser et al., 2009; Wei et al., 2012). This small effect is probably related to the opposite roles played by the major brain calpain isoforms regarding neuroprotection and neurodegeneration (Baudry & Bi, 2016). Nevertheless, the effect of TFL457 was very similarly independent of calpain inhibition. Likewise, the neuroprotective effect of TFL457 was not significantly affected by GM6001, a broad metalloproteinase inhibitor blocking the first step of RIP (Fig 6D). Thus, we suggest that the actions of TFL457 on neuronal viability occur upstream of TrkB-FL processing.

Interestingly, TFL457 could counteract actions of both proteolytic systems on TrkB-FL although the peptide contained receptor sequences away from the experimentally established calpain site (Jeronimo-Santos et al., 2015) and putative metalloproteinase targets. As mentioned, the TrkB-FL sequences included in TFL457 are inside an IDR located in a juxtamembrane region involved in regulation of receptor recycling through protein interaction (Huang et al., 2009, 2011; Zamani et al., 2018). Thus, we hypothesized that TFL457 effects on TrkB-FL stability might be indirectly mediated by a switch in recycling that places the receptor in a subcellular location where it is less susceptible to proteolysis. Cultures briefly treated with NMDA (1 h), to limit excitotoxicity-induced degradation, showed a strong decrease in cell-surface TrkB-FL levels which was similar to that described for BDNF (Fig 6E; Zheng et al., 2008). The ratio of surface to total TrkB-FL levels, which subtracts the effect of partial receptor processing, was not statistically different for NMDA- or BDNF-treated cultures. Next, we tested if TFL457 might be able to interfere the decrease in TrkB-FL cell-surface expression induced by excitotoxicity (Fig 6F). At this early time of NMDA treatment, the reduction in total receptor levels was not significantly different in TMyc or TFL457-treated cultures as expected. In contrast, surface expression of TrkB-FL was preserved after treatment with NMDA in the presence of TFL457 (98 ± 22%), differently from results obtained TMyc-treated cultures where NMDA induced a dramatic decrease (17 ± 12%). Therefore, TFL457 enhances TrkB-FL localization inside the plasma membrane after NMDAR overactivation.

Finally, we investigated the importance of TrkB-FL endocytosis for receptor proteolysis in excitotoxicity. We used a mouse model of permanent ischemia where the excitotoxic process occurs in vivo and causes profound cortex neurodegeneration as established by Fluoro-Jade C staining (Fig EV3). Human stroke is frequently caused by embolic or thrombotic occlusion of small arteries, a situation mimicked in this model by microvascular photothermolysis (Pevsner et al., 2001). Mice were injected with dynasore, to inhibit dynamin-dependent endocytosis, or vehicle before damage induction in motor and somatosensory areas (Fig 6G). Animals presented emergent cortical infarcts only 3 h after insult initiation, proving that damage is early developed in this ischemia model (Appendix Fig S3). At this early time of damage, levels of TrkB-FL and TrkB-T1 were decreased in the infarced area (I) of vehicle treated animals compared with the contralateral region (C) (Fig 6H and I, as described (Tejeda et al., 2016). However, TrkB-FL levels were significantly higher in the infarcted area of animals pretreated with dynasore compared with control animals (69 ± 21% vs. 19 ± 4%). In contrast, dynasore had no significant effect on previously described TrkB-T1 downregulation. Thus, we conclude that in vivo prevention of endocytic processes induced by ischemia results in TrkB-FL stabilization. Altogether, experiments above support that a primary mechanism of TFL457 action might be the interference of TrkB-FL endocytosis and/or enhancement of receptor recycling, resulting in a global increase in TrkB-FL levels in the membrane where the receptor might be less susceptible to proteolytic processing.

TFL457 counteracts TrkB-FL downregulation in ischemia and reduces infarct volume and neurological damage

Our next goal was to investigate if TFL457 could also interfere TrkB-FL downregulation in vivo and, therefore, act as an ischemia neuroprotectant. Preliminary experiments using a biotinylated CPP derived from a previously described neuroprotective peptide (Bio-NA-1; Aarts et al., 2002) showed correct delivery of Tat peptides to undamaged mice cortex (Fig EV4). We also demonstrated that biotinylated TFL457 (Bio-TFL457) was able to reach cortical neurons in the brain area that will be damaged in our ischemia model (Fig 7A). The in vivo analysis of TFL457 neuroprotective potential required an improvement of peptide stability in plasma. One approach is the modification of the N-ter and C-ter sequences by, respectively, acetylation and amidation to mimic the natural protein structure. In this way, we generated MTMyc and MTFL457 (Appendix Table S1). As seen before for TFL457, MTFL457 was able to interfere TrkB-FL processing in vitro (Appendix Fig S4) and also
Figure 6.
had a significant effect in vitro (Fig 7C and D). For these protection assays, the CPPs were injected after damage initiation (Fig 7B) to mimic a clinical situation where drugs would be provided after insult. The strong decrease in TrKB-FL levels observed early after damage (3 h) in the infarcted area of animals injected with MTMyc (44 ± 4%) was restrained in those treated with MTFL457 (75 ± 8%; Fig 7D). Next, we examined the long-term effects that early TrKB-FL stabilization by MTFL457 might have on infarct volume. We performed TTC staining of coronal sections obtained 24 h after brain insult (Fig 7E), a time when infarcts are well established. Interestingly, infarct volume in MTFL457-treated animals was 8 ± 0.5% of the total hemisphere volume and 11 ± 0.5% in the MTMyc group, which represents a 28% reduction after MTFL457 treatment (Fig 7F). Moreover, this neuroprotective effect correlated with an improvement in balance and motor coordination in the beam-walking test. We found a decrease in 42% in the number of slips due to MTFL457 treatment with MTMyc animals (6 ± 1 vs. 10 ± 2; Fig 7G).

In conclusion, these results demonstrate that peptide MTFL457 is neuroprotective in vivo. In a severe model of permanent brain ischemia, it reduces the infarct size and neurological damage in parallel to preservation of TrKB-FL from excitotoxicity-induced downregulation. Additionally, our data unveil the importance of this neurotrophin receptor for neuronal survival after brain damage and lead the way for the development of rational stroke therapies that efficiently support neurotrophic signaling.

Discussion

Herein, we present the design and characterization of a neuroprotective peptide able to interfere downregulation of BDNF receptor TrKB-FL induced by excitotoxicity. This effect correlates with increased viability of cortical neurons after NMDAR overactivation in vitro (acute or chronic) or in vivo (ischemic insult). Peptide TFL457 has a short TrKB-FL juxtamembrane region (aa 457–471) inner to an IDR, sequences involved in disease pathways now considered as therapeutic targets (Chen & Kriwacki, 2018). Also, this IDR is located in a receptor region (aa 453–536) previously defined as important for regulation of TrKB-FL endocytic recycling through Hrs binding (Huang et al, 2009). This region also interacts with other proteins such as suppressor of cytokine signaling 2 (SOCS2), which regulates TrkB trafficking (Zamani et al, 2018), c-Jun NH2-terminal kinase-interacting protein 3 (JIP3) that mediates interaction with kinesin-light chain (KLC1; Huang et al, 2011) or ubiquitin C-terminal hydrolase L1 (UCH-L1; Guo et al, 2017).

A link between TrkB trafficking and signaling is critical for neuronal functioning. Electric stimulation enhances the BDNF response of neurons by increasing cell-surface TrkB (Zhao et al, 2009). Ca2+ influx also improves BDNF responsiveness by increasing TrkB internalization into signaling endosomes (Du et al, 2003). In contrast, little is known about how NMDAR overactivation affects the levels of TrkB at the cell surface. Endocytosis is enhanced in excitotoxicity by a clathrin/dynamin-mediated mechanism preceding neuronal death in vitro (Vaslin et al, 2007) or ischemia (Vaslin et al, 2009). Here, we demonstrate that a brief excitotoxic insult induces a strong cell surface decrease in TrKB-FL previous to processing. By competing protein interactions, TFL457 might interfere excitotoxicity-induced receptor endocytosis or facilitate recycling back to the membrane. In fact, endocytosis inhibition strongly reduces TrKB-FL cleavage in ischemia, suggesting that receptor downregulation requires previous endocytosis. TFL457 efficiently prevents receptor decrease at the surface of excitotoxic neurons, exposing aa 457–471 as relevant to TrKB-FL trafficking in excitotoxicity. This switch in localization would interfere TrKB-FL proteolysis, protect prosurvival pathways, and finally lead to a reduced neuronal death.

This hypothesis implies that TrKB-FL processing occurs mostly outside plasma membrane. The major mechanism of TrKB-FL downregulation in excitotoxicity is cleavage by calpain (Tejeda et al, 2016), ubiquitous protease central to neuronal death in stroke, and excitotoxicity-associated neurodegenerative diseases (Bevers &
Neumar, 2008). The subcellular localization of calpain processing is under debate. Activation is favored near plasma and endosomal membranes (Tompa et al., 2001). Thus, cleavage of vascular endothelial cadherin (VE-cadherin) occurs in clathrin-enriched membrane domains during endocytosis (Su & Kowalczyk, 2017). Activation can also occur in microdomains with high local \([\text{Ca}^{2+}]\) or other compartments, such as Golgi membranes, nucleus, or mitochondria (Bevers & Neumar, 2008). RIP, secondary mechanism of TrkB-FL downregulation (Tejeda et al., 2016), is regulated by intracellular trafficking of the proteases (Morohashi & Tomita, 2013). Accordingly, A disintegrin and metalloproteinase (ADAM) 10 and 12 surface location is regulated by endocytosis and can be altered by disease (Stautz et al., 2012; Marcello et al., 2013). Activity of \(\gamma\)-secretase can also occur along endocytic and recycling pathways (Tarassishin et al., 2004). Further experiments will establish the precise location of TrkB processing.

The central role of calpain in cell physiology has steered strategies to block protease actions on specific substrates, avoiding generic effects (Wu & Tymianski, 2018). Thus, we have developed a neuroprotective CPP (Tat-K) that by interfering cleavage of Kinase D-interacting substrate of 220 kDa (Kidins220), a Trk and NMDAR-interacting protein, is able to preserve the activity of ERK1/2 and CREB survival pathways and the viability of neurons after in vitro excitotoxicity (Gamir-Morralla et al., 2015). In contrast, we could

**Figure 7.** TFL\(_{457}\) counteracts TrkB-FL downregulation in ischemia and reduces infarct volume and neurological damage.

A Confirmation of Bio-TFL\(_{457}\) delivery to mice cortex. Biotinylated TFL\(_{457}\) (Bio-TFL\(_{457}\), 4 nmol/g, retro-orbitally injected) was detected in coronal sections stained with NeuN and DAPI. Representative confocal microscopy images of cortical areas correspond to single sections. Scale bar, 10 \(\mu\)m.

B Timeline to analyze in vivo effects of MTMyc and MTFL\(_{457}\). Mice subjected to ischemia as before were retro-orbitally injected with CPPs (10 nmol/g) 10 min after damage initiation.

C Representative analysis of TrkB-FL at 3 h of damage. Comparison of the infarcted area (I) and the corresponding contralateral region (C) is shown.

D Quantitation of TrkB-FL levels. Means ± SEM of normalized TrkB-FL levels in the infarcted area relative to the corresponding contralateral region are presented and results analyzed by Student’s t-test (*\(P=0.021\); \(n=3\)).

E Representative 1 mm brain coronal slices stained with TTC after 24 h of insult corresponding to animals injected with MTMyc or MTFL\(_{457}\). Number of contralateral hind paw slips were measured after 24 h of damage. Data spread is presented by box and whisker plots showing interquartile range, median, minimum, and maximum values. Means (dots) ± SEM were also calculated and analyzed by Student’s t-test (*\(P=0.04\); \(n=11\)).

Source data are available online for this figure.
not prevent TrkB-FL downregulation or neuronal death with TFL$_{518}$, although it contains an experimentally established calpain site (Jeronimo-Santos et al., 2015). It is interesting that TrkB-FL-interacting protein FRS2, which anchors at pY515 nearby the cleavage site, has low affinity for short peptides compared with longer sequences which probably can adopt proper three-dimensional conformations (Zeng et al., 2014). However, the design of peptides longer than TFL$_{518}$ might confront unwanted side effects by potential displacement of adaptor proteins and aberrant signaling.

The receptor preserved by TFL$_{457}$ in excitotoxicity is not completely functional due to reduced Y515 phosphorylation, probably caused by tyrosine phosphatase Shp-2 (Src homology-2 domain-containing phosphatase-2) activation. Shp-2 is involved in a Ca$^{2+}$-induced feedback mechanism suppressing further pY515-dependent TrkB-FL activation (Rusanescu et al., 2005). In contrast, TFL$_{457}$ preserves Y816 phosphorylation and promotes neuroprotection dependent on PLC$_{Γ}$, neurotrophin-stimulated enzyme involved in neuronal survival (Yamada et al., 2002). Most PLC$_{Γ}$ effects are mediated by IP$_{3}$-promoted Ca$^{2+}$-release from internal stores (Numakawa et al., 2001) and activation of enzymes such as PKC or Ca$^{2+}$-calmodulin-regulated protein kinases (Huang & Reichardt, 2003). We propose that PLC$_{Γ}$ contributes to TFL$_{457}$ neuroprotection through activation of prosurvival TFs CREB and MEF2. The importance of PLC$_{Γ}$ for CREB activation is well established (Minicelli et al., 2002). Antidepressants increase TrkB-dependent CREB phosphorylation in neurons through PLC$_{Γ}$-signaling with no effect on Y515 phosphorylation (Rantamaki et al., 2007). Here, we observe that TFL$_{457}$ preserves in excitotoxicity the promoter activity of several genes containing CRE and MEF2 elements. The genes encoding CREB and MEF2 TFs are probably also affected by this peptide since their own promoters contain, respectively, CRE (Zhang et al., 2005) and MEF2 elements (Cripps et al., 2004). Interestingly, TFL$_{457}$ has also an impact on the mRNA levels of prosurvival genes regulated by these TFs (Bdnf, Ntrk2, Grin1, and Grin2a) counteracting the effects of excitotoxicity. This suggests that this peptide starts a positive auto-regulatory mechanism that highly potentiates its neuroprotective efficacy at early times and might be also critical for long-term neuronal regeneration.

TFL$_{457}$ might be highly relevant to human stroke therapy since it efficiently prevents TrkB-FL downregulation, and reduces infarct size and neurological damage in a severe model of permanent ischemia. The developed CPP provides a promising alternative/complement to the use of BDNF to recover aberrant BDNF/TrkB signaling pathways after brain damage. Although this neurotrophin has been considered for the treatment of neurological and psychiatric disorders, clinical trials with BDNF have been unsuccessful (Tejeda & Diaz-Guerra, 2017). One reason might be that the type of neuronal death induced by NMDAR overactivation (Ankarcrona et al., 1995) determines the cell response to BDNF. Using neocortical cultures (neurons and glial cells), we have confirmed previous observations demonstrating that BDNF fails to protect neurons from excitotoxicity produced by necrotic mechanisms (Koh et al., 1995). In contrast, BDNF could attenuate apoptotic neuronal death induced in these same cultures by different stimuli (Gawg et al., 1997) or in an alternative cellular paradigm of excitotoxicity (semi-pure hippocampal neurons; Gomes et al., 2012; Lau et al., 2015) where NMDA activates apoptosis (Almeida et al., 2005). Since both types of neuronal death occur in the ischemic brain, the in vivo efficacy of BDNF administration might be limited to a subpopulation of the degenerating neurons. Another challenge to the use of BDNF in brain therapy is aberrant function of TrkB-FL receptor, already described in stroke and other disorders, which might be prevented by neuroprotective peptide TFL$_{457}$.

Until now, most CPP-based strategies for stroke have targeted signaling cascades related to NMDARs (Wu & Tymianski, 2018). Recently, a Tat peptide containing GluN2B C-ter (NA-1; Aarts et al., 2002) has provided the first strong evidence that neuroprotection is feasible in human ischemic stroke (Hill et al., 2012). NA-1 dissociates GluN2B-PSD-95-nNOS complexes, uncoupling NMDARs from nNOS neurotoxic signaling, and is currently under Phase 3 clinical trials (FRONTIER and ESCAPE-NA-1). Other NMDAR-targeted CPPs under development disrupt p53 interaction with death-associated protein kinase 1 (DAPK1; Pei et al., 2014), prevent nuclear translocation of PTEN (phosphatase and tensin homolog deleted on chromosome 10; Zhang et al., 2013), or selectively reduce mGluR1 calpain processing (Xu et al., 2007). The novelty of the approach presented here is that TFL$_{457}$ targets a different signaling cascade, likewise critical to neuronal survival: the BDNF/TrkB pathway. By affecting TrkB-FL trafficking, TFL$_{457}$ blocks receptor processing and allows sustained survival signaling. Remarkably, TFL$_{457}$ has additional effects on NMDAR-dependent cascades and, therefore, has a double action on these major survival pathways, converging in CREB activation. Thus, TFL$_{457}$ might also improve endogenous neurogenesis (Lonze & Ginty, 2002) and promote functional recovery of ischemic brain. The versatility of TFL$_{457}$ could be further increased by combination with other neuroprotective CPPs, an approach suggested by the complexity of the ischemic process. Thus, if proven efficient also in vivo, peptide Tat-K (Gamir-Morralla et al., 2015) could be used simultaneously with TFL$_{457}$ to block defective neurotrophic support and NMDAR signaling at different levels. As mentioned before, another interesting possibility would be the combined action of TFL$_{457}$ with BDNF mimetic or strategies to increase BDNF availability (Tejeda & Diaz-Guerra, 2017). In summary, the stabilization of receptor TrkB-FL by the neuroprotective peptide TFL$_{457}$ has a great potential to recover TrkB and NMDAR-dependent neuroprotective pathways not only in acute stroke but also in other neurological conditions associated with excitotoxicity.

Materials and Methods

All reagents, materials, mouse models, and software used in the study are described in Appendix Table S1.

Experimental models

All animal procedures were performed in compliance with European Union Directive 2010/63/EU and were approved by the CSIC and Comunidad de Madrid (Ref PROEX 221/14) ethics committees. The housing facilities at the Institute were approved by Comunidad de Madrid (ES 280790000188) and comply with official regulations. Animals had a standard health and immune status and were looked after by professional caregivers, being checked daily. Male mice were kept in groups of up to five in standard IVC cages while 1–2 pregnant rats occupied standard cages, all of them containing bedding and nesting material. They were under controlled lighting.
conditions (12 h light cycles), relative humidity and temperature, irradiated food and water provided ad libitum. All efforts were made to minimize animal suffering and reduce the number of animals sacrificed.

**Mice model of ischemia by photothrombosis**

Permanent focal ischemia was induced in the cerebral cortex of adult male Balb/c/OlaHsd mice (25–30 g; 8–12 weeks of age; Harlan Laboratories, Boxmeer, The Netherlands) by microvascular photothrombosis. The model mimics embolic or thrombotic occlusion of small arteries, frequently found in human stroke, and causes a focal brain damage with histological and MRI correlations to human patterns (Pevsner et al, 2001). Upon receipt, animals were allowed to acclimatize to our facilities for at least 1 week prior to ischemic induction, kept with the same cage mates, and daily inspected to check normal health status. Mice were anesthetized after induction, right after irradiation completion. These slices were sectioned into serial 1-mm-thick coronal slices using a mouse brain matrix as before. Slices were then completely stained with 2% TTC at room temperature and fixed in 4% paraformaldehyde before scanning of both rostral and caudal sides.

**Primary culture of rat cortical neurons**

Primary neuronal cultures were prepared from the cerebral cortex of 18-day-old Wistar rat embryos (E18), both genders being indistinctly used. Dissected cerebral cortices were mechanically dissociated in culture medium (Minimum Essential Medium, Life Technologies, cat#21090-022) supplemented with 22.2 mM glucose, 0.1 mM glutamate, 5% fetal bovine serum, 5% donor horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin similarly as described before (Choi, 1985; Choi et al, 1987). The cell suspension was seeded at a density of 1 × 10^6 cells/ml in the same medium using plates previously treated with poly-L-lysine (100 μg/ml, Sigma-Aldrich) and laminin (4 μg/ml, Sigma-Aldrich) overnight at 37°C. Unless otherwise indicated, glial growth was inhibited after 7 days DIVs by adding cytosine β-D-arabinofuranoside (AraC, 10 μM) and experimental treatments took place after 13 DIVs.

**Induction of neuronal excitotoxicity**

To induce chronic excitotoxicity, cultures were incubated with NMDA (100 μM, unless otherwise stated) and its co-agonist glycine (10 μM), a treatment herein denoted simply as NMDA. The co-agonists induce a strong excitotoxic response in the mature neurons present in the culture but have no effect on astrocyte viability (Choi, 1985; Choi et al, 1987). When indicated, primary cultures were preincubated for 30 min with Tat-derived CPPs (> 95% purity; GenScript; 25 μM, unless otherwise indicated) or BDNF (100 ng/ml) before NMDA treatment. Peptides or BDNF were kept in the medium along treatment. For protease inhibition, cultures were preincubated with the indicated compounds (10 μM CIHl, 10 μM calpeptin, or 10 μM GM6001) for 30 min before peptide preincubation and NMDA addition, the inhibitor remaining in the medium for the duration of treatment. Enzymatic activities downstream TrkB activation were inhibited with selective inhibitors Wortmannin (100 nM), UO126 (300 nM), or U-73122 (5 μM), blocking, respectively, PI3K, MAPK/ERK, or PLCγ1, added to culture media 30 min before peptide preincubation and induction of excitotoxicity as before. KG-501 (10 μM), an inhibitor of the interactions established by the co-activator CBP with different transcription factors, including CREB, was used similarly.

To induce acute excitotoxicity, primary cultures were treated with NMDA (50 μM) and glycine (10 μM) for 1 h. Cells were then washed and fed with conditioned medium without NMDAR agonists but containing the generic antagonist DL-AP5 (200 μM), together with peptides TMyc or TFII457 (15 μM). The effect of acute NMDA treatment was analyzed 20 h later.

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Assessment of neuronal injury in cultures

We used the MTT reduction assay to measure cell viability. At the end of treatments, MTT (0.5 mg/ml, Sigma-Aldrich) was added to the medium and, after 2 h of incubation at 37°C, the formazan salts formed were solubilized in DMSO and spectrophotometrically quantified at 570 nm. As primary cultures contain neurons and glial cells, we established the contribution of glial viability to total values by exposing sister cultures to 400 μM NMDA, 10 μM glycine for 2 h before MTT assay. These conditions induce nearly complete neuronal death and no glial damage. After subtracting this absorbance value, we obtained the viability of the neuronal subpopulation. All experiments included sample triplicates for each treatment, and multiple independent experiments were carried out as detailed in the figure legends. Generally, the viability of neurons preincubated with a peptide and subjected to excitotoxicity was calculated relative to that of neurons preincubated with the same peptide but no NMDA. However, for some experiments, neuronal viability was expressed relative to the untreated cultures or those preincubated with control peptide TMyc but no NMDA.

Western blot (WB) analysis

Cultures and brain tissue were lysed in RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 1 mM DTT, and 0.1% SDS for cultures or 1% SDS for tissue) containing protease and phosphatase inhibitors (complete protease and PhosSTOP phosphatases inhibitor cocktail tablets, Roche). Protein concentration was established with BCA Protein Assay Kit (Thermo Fisher). Total cell lysates were denatured in SDS-sample buffer followed by heating at 95°C for 5 min. Equal amounts of total cell lysates were resolved in Tris-Glycine SDS–PAGE and transferred on to a nitrocellulose membrane (GE Healthcare). Membranes were stained with a Ponceau S solution to check for protein transference. After blocking with a 5% nonfat dry milk solution in Tris-buffered saline (TBS) with 0.05% Tween-20, membranes were incubated overnight at 4°C with primary antibodies and then washed and incubated with appropriate anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology or Bethesda) for 1 h at room temperature. Finally, immunoreactivity was visualized using Clarity Western ECL Blotting Substrate (BioRad) and band intensity was quantified by densitometric analysis (ImageJ). The levels of the protein of interest were normalized using those of neuron-specific enolase (NSE) present in the same sample and expressed relative to values obtained in their respective controls, arbitrarily given a 100% value. NSE was used as a neuronal loading control since it is not affected by NMDA treatment. In contrast, the activation of calpain induced by excitotoxicity was confirmed by analyzing the formation of characteristic breakdown products (BDPs; 150 and 145 kDa) from spectrin, a standard substrate of this protease. Multiple independent experiments were carried out and quantitated as detailed in the figure legends.

Immunoprecipitation

Total cell lysates were prepared from cortical cultures in 1% NP-40, 80 mM NaCl, 20 mM EDTA, and 20 mM Tris–HCl (pH 8), containing protease and phosphatase inhibitors as before. Cleared lysates (1 mg) were precipitated overnight at 4°C with 4 μg of an anti-phosphotyrosine-specific antibody (Appendix Table S1) before addition of 60 μl of 50% Protein G Agarose, incubated for 1 h at room temperature with agitation. Equal amounts of lysates or equivalent volumes of immunoprecipitated complexes were analyzed by WB as indicated.

Biotinylation of cell-surface proteins

After treatment, cultures were immediately washed with ice-cold PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂. Surface proteins were biotinylated for 30 min at 4°C with 0.5 mg/ml of NHS-SS-biotin (Thermo Fisher) prepared in the same buffer. Free biotin excess was eliminated washing cultures with cold PBS containing 1 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% BSA, followed by two additional washes omitting BSA. Lysis was in RIPA buffer with protease and phosphatase inhibitors but without DTT. Except for a total TrkB-FL aliquot, extracts were incubated with streptavidin resin (GenScript) for 3 h at 4°C to precipitate biotinylated proteins. After washing the streptavidin–biotin complexes twice with lysis buffer containing 500 mM NaCl and protease and phosphatase inhibitors, plus two additional washing steps omitting the NaCl, pellets were solubilized and denature in SDS–PAGE sample buffer (10 min at 50°C). Equivalent volumes of isolated proteins and total extracts were analyzed by WB in parallel. Independent experiments were carried out and quantified as detailed in figure legends.

Peptide visualization in primary cultures

Cultures grown on coverslips (DIV 13) were incubated for 1 h with biotin-conjugated TMyc (Bio-TMyc, 25 μM, GenScript, Appendix Table S1) or left untreated. Cells were fixed with 4% paraformaldehyde in PBS (30 min) and blocked and permeabilized with 1% BSA and 0.1% Triton X-100 in PBS (30 min). Coverslips were incubated with anti-NeuN (3 h) followed by secondary antibodies conjugated to Alexa Fluor 546, Fluorescein Avidin D (50 μg/ml), and DAPI (5 μg/ml). Mounting was in Prolong Diamond. Confocal images were acquired using an inverted Zeiss LSM 710 laser confocal microscope (Jena, Germany) with a 40× (quantitation) or 63× Plan-Apochromatic oil immersion objective (details of entry) and were normalized for each color separately. Images respectively corresponded to maximum intensity projections or single sections and were processed for presentation with ImageJ (NIH Image). A minimum of 100 neurons were counted in five independent experiments. Mean ± SEM is given.

Peptide visualization in brain cortex

Mice retro-orbitally injected with vehicle (saline), biotin-labeled NA-1 (Bio-NA-1), or TFL457 (Bio-TFL457; Appendix Table S1), used at 4 nmol/g, were deeply anesthetized 30 min after peptide administration and intracardially perfused with cold PBS and 4% paraformaldehyde in PBS. Brains were post-fixed in the same fixative at 4°C for 24 h and cryoprotected in 30% sucrose for 48 h at 4°C. Coronal frozen sections (30 μm thick) obtained using a cryostat (Leica, Heidelberg, Germany) were incubated in blocking solution (10% goat serum, 0.5% Triton X-100 in PBS) for 3 h at room temperature, followed by anti-NeuN in 4% goat serum (1:500) for 3 h. After washing, sections were incubated 1 h with Alexa Fluor...
546-conjugated antibodies (1:500), Fluorescein Avidin D (200 µg/ml), and DAPI (5 µg/ml) prepared as before. Sections were mounted and dried on slides, and cover slipped with Prolong Diamond. Tile image acquisition was performed using an inverted laser confocal microscope as before with a Zeiss LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC M27 objective (1.2× zoom). Other images were obtained with a 40× objective. Images were processed as described and correspond to single sections. Background was subtracted using vehicle-injected animals.

**Fluoro-Jade C staining**

The infarcted tissue present in the cerebral cortex of mice sacrificed 5 h after photothrombosis was first identified by Nissl staining of brain coronal sections. Specific labeling of degenerating neurons and cell nuclei was then performed in adjacent sections by Fluoro-Jade C staining. To that, sections were immersed in a basic alcohol solution (1% NaOH in 80% ethanol) for 5 min and rinsed for 2 min in 70% ethanol and for two additional min in distilled water. Next, they were incubated in a 0.06% potassium permanganate solution for 10 min, rinsed for 2 min in distilled water, and for 10 min to a fresh solution of 1 µg/ml Fluoro-Jade C (Millipore) in 0.1% acetic acid containing 0.5 µg/ml DAPI. Sections were then washed in distilled water, mounted on slides, allowed to dry overnight at room temperature, and cover slipped with Prolong Diamond. Fluorescent images were acquired using an Eclipse 90i Nikon microscope and a DS-Q1Mc Nikon digital camera. Pictures were processed with NIS-Elements 3.001, ImageJ (NIH Image), and Adobe Photoshop CC software (Adobe Systems Inc.).

**Cell transfection and gene reporter assays**

Neurons were transfected with indicated plasmids (Appendix Table S1) in neurobasal medium using Lipofectamine 2000 according to manufacturer instructions. The plasmids contained different gene promoter sequences upstream of the firefly luciferase reporter gene. After 2 h, DNA-liposomes complexes were removed and neurons fed with conditioned medium collected before. Treatments with drugs and peptides were started after completing 24 h of transfection. Lysis was in Passive Lysis Buffer (Promega, Cat# E1941), and luciferase activity was determined in 25 mM glycylglycine, 15 mM SO4Mg, 4 mM EGTA, 15 mM potassium phosphate (pH 7.8), 3.3 mM ATP, 1 mM DTT, and 75 µM luciferin. Independent experiments, including quadruplicated samples, were repeated a minimum of five times as indicated.

**RNA extraction and real-time PCR analysis**

Total RNA extracted using QIAcube technology was treated with DNases before cDNA synthesis using a “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystems). A 7900HT Fast real-time PCR system (Applied Biosystems) was used for SYBR green gene expression assays with indicated primer sequences (Appendix Table S1). A specific standard curve was performed in parallel for each gene, and technical triplicates were prepared for each sample. PCR conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Data from eight independent experiments were analyzed and normalized to levels of housekeeping genes NSE (genes of neuronal expression) or GAPDH (genes expressed in neurons and glial cells).

**Measurement of infarct volume**

Rostral and caudal images of TTC-stained coronal slices were analyzed using ImageJ software by an observer blinded to experimental groups. After image calibration, delineated areas of ipsilateral and contralateral hemispheres, and the infarcted region (unstained area) were measured. Considering slices thickness, the corresponding volumes were calculated and corrected for edema’s effect, estimated by comparing total volumes of hemispheres. The corrected infarct volumes were expressed as percentage relative to the contralateral hemisphere, to correct for normal size differences between different animals. For each animal, the mean of results obtained for rostral and caudal sides was calculated.

**Beam-walking test**

Motor coordination and balance were evaluated in mice right before and 24 h after the ischemic insult by measuring the number of contralateral hind paw slips in the beam walk apparatus. Mice had to walk through a narrow beam (1 m × 1 cm × 1 cm) placed 50 cm above the tabletop, going from an aversive stimulus (60-W light bulb) to a black goal box with nesting material. Slips taking place in a previously selected central beam segment (50 cm long) were counted. Before damage induction, mice were allowed to cross the beam once, to get acquainted with the test, which they repeated 24 h after photothrombosis, immediately before sacrifice.

**Quantification and statistical analysis**

All data were expressed as mean ± standard error of the mean (SEM) of at least three independent experiments. The details of the number of experiments done, the precise sample size, and the specific statistical test applied for each case can be found in the figure legends. For *in vivo* studies, animals were randomly allocated to the experimental groups and the researchers doing the experiments were blind respect to treatment. In order to reduce subjective bias, we used a numerical key for blinded measurement of infarct size or behavioral assessment. There was not a previous estimation of sample size, and experiments were independently carried out as indicated. We pre-established an exclusion criteria for values more than three standard deviations outside the mean, considered as outliers. For the *in vitro* studies, cell samples in each independent experiment were sister primary cultures grown in multiwell plates (whenever possible), always obtained from the same suspension of cortical cells. Treatments were assigned in a random way, and biological replicates (*n* = 3 or *n* = 4) were included for most experiments as indicated. Multiple independent experiments were then carried out as detailed in the figure legends. Statistical significance was determined by unpaired Student’s *t*-test, one-way ANOVA followed by Tukey’s HSD post hoc test, or two-way ANOVA followed by post hoc Bonferroni. Data were represented as percent of controls or maximum values as indicated. The box and whisker plot displays the data spread and five-number summary (minimum, first quartile, median, third quartile, and maximum) together with the mean value for each set of data. A *P*-value smaller than 0.05
The paper explained

Problem
Stroke is a great health and social problem worldwide. It is the second leading cause of death, the major cause of adult disability, and the second of dementia. Pharmacological therapies for ischemic stroke (85% of cases) are still limited to thrombolytic drugs, which can be only administered to very few patients.

Results
Herein, we have developed a neuroprotective compound (peptide TFL469) directed to reduce the death of those neurons surrounding the infarct core, which initially survive stroke but die in the following hours/days. The main mechanism of this neuronal death is a process known as excitotoxicity that, among other things, inhibits the functioning of a pathway critical for neuronal survival due to the breakdown of receptor TrkB-FL. By preventing TrkB-FL cleavage, TFL469 triggers a positive auto-regulatory mechanism that highly potentiates its neuroprotective efficacy and increases the levels of several proteins important for survival of neurons. This neuroprotective peptide could be highly relevant for stroke therapy since, in a mouse model of ischemia, it similarly prevents TrkB-FL downregulation, efficiently decreases the infarct size, and improves balance and motor coordination in a neurological test.

Impact
This neuroprotective peptide might be efficient in minimizing the effects of stroke but also other acute or chronic neurological disorders which are also associated with excitotoxicity, such as several neurodegenerative diseases.

was considered statistically significant (\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)). Statistical analysis was performed with the Statistical Package for Social Science (SPSS, v.19, IBM). To select the correct statistical test, data were analyzed before by SPSS for outliers, normal distribution (Kolmogorov–Smirnov and Shapiro–Wilk’s tests), and homogeneity of variances (Levene’s tests).

Expanded View for this article is available online.

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Author contributions
Conceptualization, MD-G and GST; Methodology, MD-G, GST, and GME-O; Formal Analysis, MD-G, GST, ESA, OGV, and GME-O; Investigation, GST, GME-O, OGV, and ESA; Writing—Original Draft, MD-G; Writing—Review & Editing, MD-G, GST, and GME-O; Visualization, MD-G and GST; Supervision, MD-G; Funding Acquisition, MD-G.

Conflict of interest
The authors declare that they have no conflict of interest.

References
Aarts M, Liu Y, Liu B, Bessho S, Arundine M, Gurd JW, Wang YT, Salter MW, Tymianski M (2002) Treatment of ischemic brain damage by perturbing NMDA receptor-PSD-95 protein interactions. Science 298: 846–850
Adamec E, Mohan P, Vonsattel JP, Nixon RA (2002) Calpain activation in neurodegenerative diseases: confocal immunofluorescence study with antibodies specifically recognizing the active form of calpain 2. Acta Neuropathol 104: 92–104
Allen SJ, Wilcock GK, Dawbarn D (1999) Profound and selective loss of catalytic TrkB immunoreactivity in Alzheimer’s disease. Biochem Biophys Res Commun 264: 648–651
Almeida RD, Manadas BJ, Melo CV, Gomes JR, Gräos MM, Carvalho RF, Carvalho AP, Duarte CB (2005) Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. Cell Death Differ 12: 1329–1343
Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15: 961–973
Bai G, Zhuang Z, Liu A, Chai Y, Hoffman PW (2003) The role of the RE1 element in activation of the NR1 promoter during neuronal differentiation. J Neurochem 86: 992–1005
Bamji SX, Rico B, Kimes N, Reichardt LF (2001) BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions. J Cell Biol 174: 289–299
Baudry M, Bi X (2016) Calpain-1 and calpain-2: the Yin and Yang of synaptic plasticity and neurodegeneration. Trends Neurosci 39: 235–245
Berretta A, Tzeng Y-C, Clarkson AN (2014) Post-stroke recovery: the role of activity-dependent release of brain-derived neurotrophic factor. Expert Rev Neurother 14: 1335–1344
Bevers MB, Neumar RW (2008) Mechanistic role of calpains in posts ischemic neurodegeneration. J Cereb Blood Flow Metab 28: 655–673
Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286: 1358–1362
Chen J, Kriwacki RW (2018) Intrinsically disordered proteins: structure, function and therapeutics. J Mol Biol 430: 2275–2277
Choi DW (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci Lett 58: 293–297
Choi DW, Maulucci-Gedde M, Kriegstein AR (1987) Glutamate neurotoxicity in cortical cell culture. J Neurosci 7: 357–368
Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1: 623–634
Cripps RM, Lovato TL, Olson EN (2004) Positive autoregulation of the Myocyte enhancer factor-2 myogenic control gene during somatic muscle development in Drosophila. Deu Biol 267: 536–547
Danelon V, Montroull LE, Unsain N, Barker PA, Masco DH (2016) Calpain-dependent truncated form of TrkB-FL increases in neurodegenerative processes. Mol Cell Neurosci 75: 81–92
Deogracias R, Espilguero G, Iglesias T, Rodriguez-Peña A (2004) Expression of the neurotrophin receptor trkB is regulated by the cAMP/CREB pathway in neurons. Mol Cell Neurosci 26: 470–480
Desai A, Turetsky D, Vasudevan K, Buonanno A (2002) Analysis of transcriptional regulatory sequences of the N-methyl-D-aspartate receptor...
2A subunit gene in cultured cortical neurons and transgenic mice. J Biol Chem 277: 46374–46384
Du J, Feng L, Zaitsev E, Je HS, Liu XW, Lu B (2003) Regulation of TrkB receptor tyrosine kinase and its internalization by neuronal activity and Ca\(^{2+}\) influx. J Cell Biol 163: 385–395
Dunker AK, Silman I, Uversky VN, Sussman JL (2008) Function and structure of inherently disordered proteins. Curr Opin Struct Biol 18: 756–764
DuVerle DA, Ono Y, Sorimachi H, Marumitsu H (2011) Calpain cleavage prediction using multiple kernel learning. PLoS One 6: e19035
Ferrer I, Marin C, Rey MJ, Ribalta T, Goutan E, Blanco R, Tolosa E, Marti E (1999) BDNF and full-length and truncated TrkB expression in Alzheimer disease. Implications in therapeutic strategies. J Neuropathol Exp Neural 58: 729–739
Ferrer I, Krupinski J, Couteau E, Marti E, Ambrosio S, Arenas E (2001) Brain-derived neurotrophic factor reduces cortical cell death by ischemia after middle cerebral artery occlusion in the rat. Acta Neuropathol 101: 229–238
Gamir-Morrailla A, Lopez-Menendez C, Ayuso-Dolado S, Tejeda GS, Montaner J, Rossell A, Iglesias T, Diaz-Guerra M (2015) Development of a neuroprotective peptide that preserves survival pathways by preventing Kidins220/ARMS calpain processing induced by excitotoxicity. Cell Death Dis 6: e1939
Gascon S, Deogracias R, Sobrado M, Roda JM, Renart J, Rodriguez-Pena A, Diaz-Guerra M (2005) Transcription of the NR1 subunit of the N-methyl-D-aspartate receptor is down-regulated by excitotoxic stimulation and cerebral ischemia. J Biol Chem 280: 35018–35027
Gascon S, Sobrado M, Roda JM, Rodriguez-Pena A, Diaz-Guerra M (2008) Excitotoxicity and focal cerebral ischemia induce truncation of the NR2A and NR2B subunits of the NMDA receptor and cleavage of the scaffolding protein PSD-95. Mol Psychiatry 13: 99–114
Gawg Bj, Koh JY, Delmaro JA, Ying HS, Jacquim M, Choi DW (1997) Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. Neuroscience 77: 393–401
Gerencser AA, Mark KA, Hubbard AE, Divakaruni AS, Mehrabian Z, Nicholls DG, Ferrer I, Marin C, Rey MJ, Ribalta T, Goutan E, Marti E, Ambrosio S, Arenas E (2011) Delayed rescue of N-methyl-D-aspartate receptor-mediated neuronal injury in cortical culture. J Pharmacol Exp Ther 250: 752–758
Hecht M, Bromberg Y, Rost B (2015) Better prediction of functional effects for sequence variants. BMC Genom 16(Suppl 8): S1
Hill MD, Martin RH, Mikulis D, Wong JH, Silver FL, Terbrugge KG, Milot G, Clark WM, Macdonald RL, Kelly ME et al (2012) Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): a phase 2, randomised, double-blind, placebo-controlled trial. Lancet Neurol 11: 942–950
Hirata K, Kuge Y, Yokota C, Harada A, Kokame K, Inoue H, Kawashima H, Hanzawa H, Shono Y, Saij H et al (2011) Gene and protein analysis of brain derived neurotrophic factor expression in relation to neurological recovery induced by an enriched environment in a rat stroke model. Neurosci Lett 495: 210–215
Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72: 609–642
Huang SH, Zhao L, Sun ZP, Li XZ, Geng Z, Zhang KD, Chao MV, Chen ZY (2009) Essential role of Hrs in endocytic recycling of full-length TrkB receptor but not its isoform TrkB.T1. J Biol Chem 284: 15126–15136
Huang S-HH, Duan S, Sun T, Wang J, Zhao L, Geng Z, Yan J, Sun H-jj, Chen Z-Y (2011) JIP3 mediates TrkB axonal anterograde transport and enhances BDNF signaling by directly bridging TrkB with kinesin-1. J Neurosci 31: 10602–10614
Jeronomi-Santos A, Vaz SH, Parreira S, Rapaz-Lerias S, Caetano AP, Buee-Scherrer V, Castren E, Valente CA, Blum D, Sebastiao AM et al (2015) Dysregulation of TrkB receptors and BDNF function by amyloid-beta peptide is mediated by calpin. Cereb Cortex 25: 3107–3121
Jin N, Qian W, Yin X, Zhang L, Iqbal K, Grunke-Iqbal I, Gong Cx, Liu F (2013) CREB regulates the expression of neuronal glucose transporter 3: a possible mechanism related to impaired brain glucose uptake in Alzheimer’s disease. Nucleic Acids Res 41: 3240–3256
Koh JY, Gwag Bj, Lobner D, Choi DW (1995) Potentiated necrosis of cultured cortical neurons by neurotrophins. Science 268: 575–578
Kozlowski LP, Bujnicki JM (2012) MetaDisorder: a meta-server for the prediction of intrinsic disorder in proteins. BMC Bioinformatics 13: 111
Krainc D, Bai G, Okamoto S, Carles M, Kusiak JW, Brent RN, Lipton SA (1998) Synergistic activation of the N-methyl-D-aspartate receptor subunit 1 promoter by myocyte enhancer factor 2C and Sp1. J Biol Chem 273: 26218–26224
Lau GC, Saha S, Faris R, Russek SJ (2004) Up-regulation of NMDAR1 subunit gene expression in cortical neurons via a PKA-dependent pathway. J Neurochem 88: 564–575
Lau D, Bengtsson CP, Buchthal B, Bading H (2015) BDNF reduces toxic extrasynaptic NMDA receptor signaling via synaptic NMDA receptors and nuclear-calcium-induced transcription of inhba/activin A. Cell Rep 12: 1353–1366
Linseman DA, Bartley CM, Le SS, Laessig TA, Bouchard RJ, Meintzer MK, Li M, Heidenreich KA (2003) Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+)-calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. J Biol Chem 278: 41472–41481
Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z (2003) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci USA 100: 8532–8537
Liu Z, Cao J, Gao X, Ma Q, Ren J, Xue Y (2011) GPS-CCD: a novel computational program for the prediction of calpain cleavage sites. PLoS One 6: e19001
Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35: 605–623
Lyons MR, Schwarz CM, West AE (2012) Members of the myocyte enhancer factor 2 transcription factor family differentially regulate Bdnf transcription in response to neuronal depolarization. J Neurosci 32: 12780–12785

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Madinier A, Bertrand N, Rodier M, Quirin A, Mossiat C, Pingnet-Tessier A, Marie C, Garnier P (2013) Ipsilateral versus contralateral spontaneous post-stroke neuroplastic changes: involvement of BDNF? Neuroscience 231: 169 – 181.

Marcello E, Saracenoc C, Musardoc S, Varha H, de la Fuencort A, Pelucchis D, Di Marinos D, Borronoi B, Tramontanoc A, Pérez-Otañoc i et al (2013) Endocytosis of synaptic ADAM10 in neuronal plasticity and Alzheimer's disease. J Clin Invest 123: 2523 – 2538.

Minichiellio L, Calellama AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36: 121 – 137.

Morohashiy T, Tomita T (2013) Protein trafficking and maturation regulate intramembrane proteolysis. Biochim Biophys Acta 1828: 2855 – 2861.

Numakawat T, Matsumotot T, Adachin N, Yokomakud Kojimam K, Takeim N, Hatanakam H (2001) Brain-derived neurotrophic factor triggers a rapid glutamate release through increase of intracellular Ca2+ and Na+ in cultured cerebellar neurons. J Neurosci Res 66: 96 – 108.

Papadias S, Hardingham GE (2007) The dichotomy of NMDA receptor signaling. Neuroscientist 13: 572 – 579.

Pasquale V, Martinoino S, Chiappalone M (2017) Stimulation triggers endogenous activity patterns in cultured cortical networks. Sci Rep 7: 9080.

Pei L, Shang Y, Jin H, Wang S, Wei N, Yan H, Wu Y, Yao C, Wang X, Zhu LJ et al (2014) DAPK1–p53 interaction converges necrotic and apoptotic pathways of ischemic neuronal death. J Neurosci 34: 6546 – 6556.

Pevsner PH, Eichenbaum JW, Miller DC, Pivawer G, Eichenbaum KD, Stern A, Zakian KL, Koutcher JA et al (2007) A photothrombotic model of small early ischemic infarcts in the rat brain with histologic and MRI correlation. Neuroscientist 13: 572 – 579.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361: 1545 – 1564.

Rusanescu G, Yang W, Bai A, Neel BG, Feig LA (2005) Tyrosine phosphatase SHP-2 is a mediator of activity-dependent neuronal excitotoxicity. EMBO J 24: 305 – 314.

Stautz D, Leyme A, Grandal MV, Albrechtson R, van Deus B, Wewer U, Kveberg M (2012) Cell-surface metalloprotease ADAM12 is internalized by a clathrin- and Grb2-dependent mechanism. Traffic 13: 1532 – 1546.

Su W, Kowalczzyk AP (2017) The VE-cadherin cytoplasmic domain undergoes proteolytic processing during endocytosis. Mol Biol Cell 28: 76 – 84.

Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, Mao Z (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J Neurosci 25: 4823 – 4834.

Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME (1998) Ca(2+) influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20: 709 – 726.

Tarassishin L, Yin Yi, Bassit B, Li YM (2004) Processing of Notch and amyloid precursor protein by gamma-secretase is spatially distinct. Proc Natl Acad Sci USA 101: 17050 – 17055.

Tejeda GS, Ayuso-Dolado S, Arbeteta R, Esteban-Ortega GM, Vidalaurre OG, Diaz-Guerra M (2016) Brain ischaemia induces shedding of a BDNF-scavenger ectodomain from TrkB receptors by excitotoxicity activation of metalloproteinases and gamma-secretases. J Pathol 238: 627 – 640.

Tejeda GS, Diaz-Guerra M (2017) Integral characterization of defective BDNF/TrkB signalling in neurological and psychiatric disorders leads the way to new therapies. Int J Mol Sci 18: E268.

Tompa P, Emony J, Sorimachi H, Suzuki K, Friedrich P (2001) Domain III of calpain is a Ca(2+)-regulated phospholipid-binding domain. Biochem Biophys Res Commun 280: 1333 – 1339.

Tompa P, Prilusky J, Silman I, Sussman JL (2008) Structural disorder serves as a weak signal for intracellular protein degradation. Proteins 71: 903 – 909.

Vaslin A, Puyal J, Borsello T, Clarke PG (2007) Excitotoxicity-related endocytosis in cortical neurons. J Neurochem 102: 789 – 800.

Vaslin A, Puyal J, Clarke PG (2009) Excitotoxicity-induced endocytosis confers drug targeting in cerebral ischemia. Ann Neurol 65: 337 – 347.

Verspuren J, Gevaert K, Declercq W, Vandenabeele P (2009) SitePredicting the cleavage of proteinase substrates. Trends Biochem Sci 34: 319 – 323.

Vidalaurre OG, Gascon S, Deogracias R, Sobrado M, Cuadrado E, Montaner J, Rodriguez-Pena A, Diaz-Guerra M (2012) Imbalance of neurotrophin receptor isoforms TrkB-FL/TrkB-T1 induces neuronal death in excitotoxicity. Cell Death Dis 3: e256.

Watson BD, Dietrich WD, Bustro R, Wachtel MS, Ginsberg MD (1985) Induction of reproducible brain infarction by photochemically initiated thrombosis. Ann Neurol 17: 497 – 504.

Wei G, Yin Y, Li W, Bito H, She H, Mao Z (2012) Calpain-mediated degradation of myocyte enhancer factor 2D contributes to excitotoxicity by activation of extrasympathetic N-methyl-D-aspartate receptors. J Biol Chem 287: 5797 – 5805.

Woronicz JD, Lina A, Calnan BJ, Szychowski S, Cheng L, Winoto A (1995) Regulation of the Nur77 orphan steroid receptor in activation-induced apoptosis. Mol Cell Biol 15: 6364 – 6376.

Wu Q, Tymaniaks M (2018) Targeting NMDA receptors in stroke: new hope in neuroprotection. Mol Brain 11: 15.

Xu W, Wong TP, Chery N, Gaertner T, Wang YT, Baudry M (2007) Calpain-mediated mGluR1alpha truncation: a key step in excitotoxicity. Neuron 53: 399 – 412.

Yamada M, Numakawa T, Koshimizu H, Tanabe K, Wada K, Koizumi S, Hatanaka H (2002) Distinct usages of phospholipase Cγ and Shc in intracellular signaling stimulated by neurotrophins. Brain Res 955: 183 – 190.

Zafra F, Castrén E, Thoenen H, Lindholm D (1991) Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. Proc Natl Acad Sci USA 88: 10037 – 10041.

Zamani A, Xiao J, Turnley AM, Murray SS (2018) Tropomyosin-related kinase B (TrkB) regulates neurite outgrowth via a novel interaction with suppressor of cytokine signalling 2 (SOCS2). Mol Neurobiol 56: 1262 – 1275.

Zeng L, Kuti M, Mujtaba S, Zhou MM (2014) Structural insights into FRS2alpha PTB domain recognition by neurotrophin receptor TrkB. Proteins 82: 1534 – 1541.

Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, Chen H, Jenner R, Herbstheimer E, Jacobsen E et al (2005) Genome-wide analysis of CAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci USA 102: 4459 – 4464.
Zhang S, Taghibiglou C, Girling K, Dong Z, Lin SZ, Lee W, Shyu WC, Wang YT (2013) Critical role of increased PTEN nuclear translocation in excitotoxic and ischemic neuronal injuries. J Neurosci 33: 7997–8008
Zhao L, Sheng AL, Huang SH, Yin YX, Chen B, Li XZ, Zhang Y, Chen ZY (2009) Mechanism underlying activity-dependent insertion of TrkB into the neuronal surface. J Cell Sci 122: 3123–3136
Zheng J, Shen WH, Lu TJ, Zhou Y, Chen Q, Wang Z, Xiang T, Zhu YC, Zhang C, Duan S et al (2008) Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth. J Biol Chem 283: 13280–13288

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