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Brain ischemia induces shedding of a BDNF-scavenger ectodomain from TrkB receptors by excitotoxicity-activation of metalloproteinases and γ-secretases

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ABSTRACT

Stroke remains a leading cause of death and disability in the world whereas limited therapies are available to restrict brain damage or improve functional recovery after cerebral ischemia. A promising strategy currently under investigation is the promotion of brain-derived neurotrophic factor (BDNF)-signalling through tropomyosin-related kinase B (TrkB) receptors, a pathway essential for neuronal survival and function. However, TrkB and BDNF-signalling are impaired in excitotoxicity, a primary pathological process in stroke also associated to neurodegenerative diseases. Pathological imbalance of TrkB isoforms is critical to neurodegeneration and is caused by calpain-processing of BDNF high affinity full-length receptor (TrkB-FL) and inversion of the transcriptional pattern of the *Ntrk2* gene, which favours expression of the truncated isoform TrkB-T1 over TrkB-FL. We report here that both TrkB-FL and neuronal TrkB-T1 also undergo ectodomain shedding by metalloproteinases activated after ischemic injury or excitotoxic damage of cortical neurons. Subsequently, the remaining membrane-bound C-terminal fragments (CTFs) are cleaved by γ-secretases within the transmembrane region, releasing their intracellular domains (ICDs) into the cytosol. Therefore, we identify TrkB-FL and TrkB-T1 as new substrates of regulated intramembrane proteolysis (RIP), a mechanism that highly contributes to TrkB-T1 regulation in ischemia but is minor for TrkB-FL which is mainly processed by calpain. However, since the secreted TrkB ectodomain acts as a BDNF-scavenger and significantly alters BDNF/TrkB-signalling, the mechanism of RIP could contribute to neuronal death in excitotoxicity. These results are highly relevant since they reveal new targets for the rational design of therapies to treat stroke and other pathologies with an excitotoxic component.

Keywords: TrkB, excitotoxicity, ischemia, metalloproteinases, γ-secretases

INTRODUCTION

Stroke is a leading cause of mortality, adult disability and dementia. However, therapies for ischemic stroke are limited to thrombolytic drugs, administered only very early after stroke onset and contraindicated for haemorrhagic stroke and other frequent medical conditions. New treatments are being developed to restrict brain damage and improve functional recovery by reducing the secondary neuronal death in regions surrounding the infarct where excitotoxicity is a primary pathological mechanism. This form of neuronal death is induced by overstimulation of the NMDA type of glutamate receptors (NMDARs) and, interestingly, also contributes to neurodegeneration in other acute disorders (trauma, epilepsy) or chronic diseases (Alzheimer, Parkinson or Huntington) [1]. An alternative neuroprotective strategy is promotion of neuronal survival by treatment with neurotrophins [2, 3], mostly brain-derived neurotrophic factor (BDNF). However, aberrant BDNF-signalling through tropomyosin-related kinase B (TrkB) receptors has been described in excitotoxicity [4, 5], Alzheimer's disease (AD) [6, 7], other neurodegenerative diseases (NDDs) [8, 9] and stroke [4]. Thus, a rational design of neuroprotective therapies for excitotoxicity-associated pathologies requires an in-depth characterization of processes responsible for impaired BDNF/TrkB-signalling.

Neurotrophin signalling-pathways, particularly those initiated by BDNF, regulate multiple physiological processes during development and adult life. BDNF binds to the extracellular interface of its high affinity receptor full-length TrkB (TrkB-FL), a glycosylated membrane protein, and induces TrkB-FL dimerization. This process increases catalytic activity of the intracellular tyrosine-kinase (TK) domain and subsequent transphosphorylation of tyrosine residues situated outside the activation loop (Y515 and Y816), allowing adaptors recruitment [10]. This triggers interconnected signalling-pathways regulating gene expression, neurotransmitter release, synaptic transmission and neuronal survival, among others [11, 12]. Along with TrkB-FL, *Ntrk2* alternative-splicing yields truncated isoform TrkB-T1 which lacks

the TK domain and is abundantly expressed in brain [13]. TrkB-T1 opposes TrkB-FL function [14, 15] via competition for BDNF binding [13] or formation of inactive heterodimers [16]. Expression of TrkB-T1 is not restricted to neurons as TrkB-FL, but also occurs in glial cells [17, 18]. TrkB-T1 (95-kDa) contains the same extracellular region, transmembrane domain and initial intracellular 12-aminoacids as TrkB-FL (145-kDa), but has 11 conserved specific residues in the C-terminus [13, 16]. Accordingly, independent TrkB-T1-signalling has also been suggested [19].

In human stroke [4] and ischemia models [4, 5, 20], excitotoxicity triggers TrkB-T1 neurospecific upregulation and TrkB-FL decrease [4]. Recovery of TrkB-FL/TrkB-T1 balance protects neurons from excitotoxicity, demonstrating that these changes are critical for neurodegeneration [4]. Two mechanisms cooperate for TrkB dysregulation [4]: inversion of its isoform-mRNAs ratio, which favours TrkB-T1 expression over TrkB-FL, and TrkB-FL calpain-processing that generates a truncated receptor similar to TrkB-T1 which might act as an additional dominant-negative inhibitor or signal TK-independently [4, 5]. Calpain-activation is associated to ischemia and excitotoxicity [21] and mediates neuronal injury by cleavage and activity modification of its substrates [22]. Additional proteolytic families such as metalloproteinases and secretases, crucial for CNS physiology [23, 24], are also important in AD [25] or ischemia [26-28]. Since TrkB-signalling is essential in making life/death decisions, we decided to deepen previous characterization of receptor dysregulation in excitotoxicity. We found that TrkB-FL and TrkB-T1 undergo *in vitro* and *in vivo* processing by metalloproteinases within their common extracellular region, shedding a soluble receptor ectodomain that acts as a BDNF-scavenger and further alters BDNF/TrkB-signalling. The complementary membrane-anchored fragments are then cleaved by γ-secretases in a characteristic process of regulated intramembrane proteolysis (RIP). These results are highly relevant since they direct a rational

design of neuroprotective therapies aimed to decrease damage in stroke and other excitotoxicity-associated pathologies.

MATERIALS AND METHODS

Model of cerebral ischemia

Animal procedures were in compliance with European Union Directive 2010/63/EU and approved by CSIC and Comunidad de Madrid (Ref PROEX 221/14) ethics committees. Focal cortical ischemia was induced in male Balb/c mice (25-30g; Harlan Laboratories, Boxmeer, The Netherlands) aged 2-3 months by photothrombosis performed as described [30] with modifications (see supplementary materials and methods). When indicated, GM6001 (100 mg/kg) or vehicle (20% DMSO, 10% Tween-20 in saline) was injected intraperitoneally just before Rose Bengal administration.

Primary neuronal cultures

Cultures were prepared from cerebral cortex of 18-day-old Wistar rat embryos as described [29]. Lentivirus infection and glial growth inhibition with AraC (10 µM) were performed at 7 DIVs and experimental treatments after 13 DIVs.

Preparation of protein extracts

Cortical cultures and brain tissue were lysed in RIPA buffer containing protease and phosphatase-inhibitors as described [29]. For analysis of culture media, they were directly heated in SDS-sample buffer at 37°C for 15 min. Those containing serum were previously incubated during 1 h with Blue-Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK) for albumin removal. Immunoblot was performed as outlined in supplementary materials and methods.

Immunofluorescence

This procedure is outlined in supplementary materials and methods.

Immunohistochemistry

Infarcted tissue was identified by staining slide-mounted brain coronal sections with Nissl. Adjacent sections were processed for immunohistochemistry as described [4] with some modifications (see supplementary materials and methods).

TrkB ectodomain purification

HA-TrkB-T1 was expressed in human embryonic kidney 293T cells (HEK293T) by plasmid LV-HA-TrkB-T1/GFP transfection. After incubation with APMA (100 µM) for 6 h, high-abundance proteins in collected medium were depleted using Blue-Sepharose and soluble HA-fragments immunoprecipitated with Pierce HA-Tag IP/Co-IP Kit (Life Technologies) following manufacturer's instructions.

Statistical analysis

All data were expressed as mean ± SEM of at least three independent experiments. Raw data were transformed according to a described correction method [31] and statistical analysis was performed by Student's *t*-test, one-way ANOVA or repeated measures ANOVA followed by Dunnett's post-test. The latter transformation prevents the use of a control group with zero variance that can reduce the analysis power and violates the ANOVA assumption of equivalent variances [32]. Data were represented as percent of controls or maximum values as indicated. A p-value smaller than 0.05 was considered statistically significant. *$p<0.05$, **$p<0.01$, *** $p<0.001$.

RESULTS

TrkB-FL calpain-processing produces cytoplasmic fragments early after *in vitro* or *in vivo* excitotoxicity

NMDAR-overactivation induces efficient TrkB-FL calpain-processing in juxtamembrane intracellular sequences producing a truncated fragment (tTrkB-FL) similar to TrkB-T1 [4]. To

better understand this pathological process, we analysed the complementary intracellular fragment with an isoform-specific antibody for unique C-terminal sequences (TrkB-FL Ct). Cortical neurons of 13 days *in vitro* (DIVs) were incubated with high concentrations of NMDAR co-agonists, NMDA (100 μM) and glycine (10 μM), henceforth designated as NMDA (Figure 1A). This treatment induced a rapid TrkB-FL reduction (68±5% after only 30 min compared to untreated cultures; $p<0.01$; Figure 1B) and a reciprocal increase of a 32-kDa fragment (f32) concomitant to calpain-activation, established by cleavage of its well-characterized substrate spectrin (240-kDa) into breakdown products (BDPs; 150 and 145-kDa). Other neuronal proteins such as neuronal-specific enolase (NSE) were not affected. Interestingly, longer autoradiography exposure also showed formation of minor fragments of approximately 42 to 39-kDa (f42/39) (Figure 1A). The decrease of TrkB-FL was partially prevented by calpain-inhibitors III (CiIII; 10 μM) and calpeptin (Calp; 10 μM) (respectively 72±10% and 61±7% versus 32±4% without inhibitors; Figures 1C, D). A similar effect was observed on f32 production. However, calpain-inhibitors increased f42/39 levels induced by excitotoxicity (e.g. 75±17% with NMDA+CiIII compared to 18±6% after NMDA treatment), suggesting that they are produced by a different excitotoxicity-activated protease but quickly processed by calpain to produce f32. Proteasome-inhibition by lactacystin (Lact; 20 μM) did not prevent TrkB-FL downregulation or f32 and f42/39 formation but induced a slight increase in fragment levels suggesting that, once produced, they undergo partial proteasomal degradation. Immunofluorescence analysis showed that TrkB-FL is spread in neuronal dendrites and cell-bodies (Figure 1E, panel a), where it is mostly associated to membrane (panel a1). Treatment with NMDA (2 h) induced signal decrease (Figure 1E, panel b), particularly in dendrites (compare panels a2 and b2). However, levels were still high in soma although there was a remarkable decrease of membrane staining (compare panels a1 and b1), probably due to receptor-processing and f32 cytosolic accumulation. Levels of neuronal-

specific protein synapsin only showed some redistribution, reflecting characteristic morphological changes associated to excitotoxicity [33].

It was important to establish if these TrkB-FL fragments were induced by excitotoxicity *in vivo*. Human stroke is frequently caused by embolic or thrombotic occlusion of small arteries, thus we used a model of focal ischemia induced by microvascular photothrombosis [34] that presents histological and MRI correlation to patterns occurring in patients [35]. Cortical infarcts in motor and somatosensory areas, established as hypochromatic areas after TTC and Nissl staining (see supplementary material, Figure S1), were obtained from animals sacrificed at very early (2.5 and 5 h) or late (24 h) times after damage induction and analysed by immunoblot (Figure 2A). Compared to contralateral tissue or sham-operated animals, TrkB-FL dramatically decreased very early in the infarcted region (Figure 2B; 20±2% at 2.5 h; $p<0.05$; n=3), concurrent with calpain-activation and accumulation of fragments similar in size and relative abundance to f32 and f42/39 (Figure 2A). Maximum f32 levels were obtained at 5 h of injury (100%), although they were already significantly increased at 2.5 h (Figure 2C; 53±4%; $p<0.001$). Regarding f42/39, maximum levels were observed at 2.5 h although differences were not statistically significant. TrkB-FL proteolysis and calpain-activation were delayed in mice with lower susceptibility to brain ischemia (see supplementary material, Figure S2), suggesting a dependence on injury severity. Immunohistochemistry confirmed a moderate decrease of TrkB-FL in degenerating neurons of ischemic tissue identified by neurodegeneration specific marker Fluoro-Jade C (Figure 2D). TrkB-FL signal was better preserved in neuronal cell-bodies (arrowheads) possibly due to intracellular fragments detection. Altogether, these results confirm calpain-processing as a critical mechanism of TrkB-FL downregulation induced by *in vitro* and *in vivo* excitotoxicity, producing a C-terminal cytosolic fragment of 32-kDa complementary to the truncated receptor previously described

[4]. TrkB-FL is also substrate of an alternative proteolytic system which produces minor 42 to 39-kDa intracellular fragments that must contain the calpain-cleavage sequence.

TrkB-FL is processed by excitotoxicity-activated metalloproteinases and γ-secretases generating a receptor ectodomain and transient intracellular fragments

The discovery of a novel TrkB-FL regulatory mechanism encouraged us to search for the responsible protease/s. The size of minor fragments suggested receptor-processing within juxtamembrane regions by RIP, an essential CNS mechanism for cell communication altered in pathologies such as AD [25]. This sequential two-steps mechanism usually acts on single-pass transmembrane receptors which are first cleaved by metalloproteinases in extracellular juxtamembrane residues in a process called ectodomain shedding that generates a soluble extracellular domain (ECD) [36]. It also produces a membrane-anchored C-terminal fragment (CTF) that can be further processed by γ-secretases in intramembrane sequences, releasing an intracellular domain (ICD) to the cytosol [23, 24]. To test this hypothesis, neurons were preincubated with specific metalloproteinases or γ-secretases-inhibitors, respectively TAPI-2 (10 μM) or Compound E (Comp E; 1 μM), before NMDA treatment (6 h, Figure 3A). Formation of f42/39 in excitotoxicity was strongly prevented by metalloproteinases-inhibition, while γ-secretases-blockage caused enrichment of the larger polypeptides in f42/39. To analyse the relative contribution of this new mechanism to TrkB-FL downregulation, we inhibited metalloproteinases and/or calpain before NMDA treatment (Figure 3B). TAPI-2 did not affect major TrkB-FL processing by calpain and f32 was similarly produced in cultures treated with NMDA with or without TAPI-2 (respectively 108±7% and 100±7%, n=6). Thus, metalloproteinases inhibition only had a minor effect on TrkB-FL recovery, confirming that RIP contribution to TrkB-FL regulation is quantitatively minor. A combination of metalloproteinases and calpain-inhibitors decreased f42/39 and f32 and caused a substantial but incomplete TrkB-FL recovery in excitotoxicity (Figure 3B), reflecting the contribution of still

additional mechanisms such as transcriptional dysregulation [4]. Calpain-inhibition confirmed that short-lived f42/39 are calpain substrates and revealed that metalloproteinases and calpain-activation occur in the same cells. TrkB-FL cleavage by these proteolytic systems was similarly induced by NMDAR-overactivation in the presence of BDNF (100 ng/ml), which did not interfere TrkB-FL downregulation and inactivation (see supplementary material, Figure S3).

We next analysed cultured media (CM) together with cell-lysates (CLs) of neurons preincubated with TAPI-2 or Comp E and NMDA treated as before (Figure 3C). Excitotoxicity induced the release of a soluble 74-kDa TrkB-ECD or ectodomain, detected with a panTrkB antibody for extracellular regions present in all isoforms. Metalloproteinases-inhibition strongly prevented ECD accumulation in CM along with f42/39 in CLs, while γ-secretases-blockage had no effect on ECD and only caused f42 enrichment. Then, we compared the effects of NMDA (Figure 3D, F) or APMA (100 μM; Figure 3E, G), used to stimulate latent metalloproteinases [37] without calpain-activation, and found statistically significant release of TrkB-ECD from early times of treatment that further increased afterwards. Ectodomain shedding was paralleled by TrkB-FL decrease, slower in APMA (Figure 3E) compared to NMDA treated cultures (Figure 3D). To unequivocally establish that TrkB-FL shedding contributes to total ECD accumulation, we analysed cultures infected with LV-HA-TrkB-FL/GFP [4] a dual lentivirus that neurospecifically expresses a recombinant N-terminal HA-tagged TrkB-FL and GFP (Figure 3H). Shedding of HA-TrkB-FL extracellular fragments (HA-ECD) was induced by NMDA (4 h) and prevented by broad metalloproteinases-inhibitor GM6001 (10 μM; Figure 3I). Treatment with APMA similarly induced HA-ECD shedding and TrkB-FL-CTF accumulation (Figure 3J). In conclusion, these results reveal TrkB-FL as a novel substrate of RIP which is induced by excitotoxicity. Although representing a minor contribution to TrkB-FL downregulation, ectodomain shedding might critically interfere BDNF/TrkB-signalling.

TrkB-T1 is sequentially processed by metalloproteinases and γ-secretases in excitotoxicity releasing its ectodomain and a short ICD

The truncated isoform TrkB-T1 has identical ECD, transmembrane segment and first intracellular 12-aminoacids to TrkB-FL so it might also undergo excitotoxicity-induced RIP. To test this hypothesis, we first tried to visualize the putative endogenous TrkB-T1 fragments formed after NMDAR-overactivation by isoform-specific antibodies for unique C-terminal sequences. Excitotoxicity induced a moderate increase of TrkB-T1 (171±20% after 6 h versus 100% for untreated neurons, n=6 $p<0.05$; Figure 4A) as described [4], similarly observed by immunofluorescence (Figure 4B). However, we could not detect any intracellular polypeptide indicative of metalloproteinase-processing. Only after direct metalloproteinases-activation by APMA, we identified limited amounts of a peptide of approximately 10-kDa (f10) which might correspond to TrkB-T1-CTF (Figure 4C) and significantly accumulated along treatment (Figure 4D). To avoid limitations due to small size and low levels of endogenous intracellular fragments, we relied again on lentivirus vectors for neurospecific expression of a recombinant TrkB-T1 fused to GFP at the C-terminus and with an HA-tag at the N-terminus (LV-HA-TrkB-T1-GFP, Figure 4E). NMDA treatment moderately decreased HA-TrkB-T1-GFP (HA-T1-GFP) levels in parallel to the accumulation of GFP-containing peptides of 34 and 30-kDa which might correspond respectively to GFP-fused TrkB-T1-CTF (CTF-GFP) and ICD (ICD-GFP; Figure 4F). The 27-kDa peptide was identified as GFP by comparing cultures infected with LV-HA-TrkB-T1-GFP or control virus LV-GFP [38] (see supplementary material, Figure S4). Interestingly, we also observed release of an HA-ECD similar to that described for TrkB-FL (Figure 4F). Since metalloproteinases-inhibition secondarily blocks activity of subordinated γ-secretases, formation of all three HA-T1-GFP fragments was prevented by GM6001. In contrast, direct γ-secretase-inhibition restrained ICD-GFP generation induced by NMDA while it did not alter the metalloproteinases products (Figure 4G). Finally, metalloproteinases-

activation with APMA demonstrated RIP of recombinant (HA-T1-GFP) and endogenous TrkB-T1 (T1) proteins (Figure 4H). Altogether, these results demonstrate neuronal RIP of TrkB-T1 in excitotoxicity. Similarly to TrkB-FL, this mechanism probably represents a small contribution to TrkB-T1 regulation in excitotoxicity *in vitro* but the properties of the generated fragments might have a significant impact in neuronal death.

TrkB-FL and TrkB-T1 are processed by metalloproteinases in brain ischemia

To establish if TrkB-T1 regulation by metalloproteinases also takes place *in vivo*, as previously found for TrkB-FL (Figure 2), we analysed the truncated isoform in mice subjected to ischemia or sham-operated (Figure 5A). In the infarcted region, TrkB-T1 levels decreased early after injury compared to contralateral tissue (Figure 5B; 54±8% at 2.5 h, $p<0.01$; n=3), while there was a progressive accumulation of a TrkB-T1-CTF statistically significant from 5 h of damage ($p<0.05$; Figure 5C). TrkB-T1 decrease, which apparently contradicts results obtained *in vitro* (Figure 4A and [4]), probably reflects *in vivo* preponderance of TrkB-T1 proteolytic over transcriptional regulatory mechanisms. Confocal microscopy of coronal sections of mice subjected to ischemic damage (5 h) showed increased TrkB-T1 signal in degenerating neurons of ipsilateral hemisphere (Figure 5D). Similar increases were described before in a rat model of middle cerebral artery occlusion and human stroke necropsies and might be due to epitope unmasking of TrkB-T1-CTF and ICD. Actually, epitope recognition can be interfered by protein-protein associations established at this unique TrkB-T1 sequence [39].

Next, we confirmed that TrkB-FL f42/39 (Figure 2D) and TrkB-T1-CTF formed *in vivo* were the result of metalloproteinase-processing. Mice were injected with GM6001 (100 mg/kg, i.p., n=4/group) or vehicle just before Rose Bengal administration and then sacrificed 3 h after damage induction (Figure 5E). In infarcted tissue, we observed a significant decrease of those fragments upon metalloproteinases-inhibition compared to control animals (Figure 5F),

reaching values of 24±5% (Figure 5H; $p<0.001$) and 4±1% (Figure 5J; $p<0.001$) respectively for f42/39 and TrkB-T1-CTF. Additionally, this drug did not modify TrkB-FL downregulation (Figure 5G) or f32 formation (data not shown) induced by ischemia, supporting a minor contribution of metalloproteinases compared to calpain in TrkB-FL regulation, while it reverted TrkB-T1 decrease (Figure 5I). In conclusion, these results demonstrate that metalloproteinase-processing is an early mechanism of TrkB-FL and TrkB-T1 regulation induced by brain ischemia, more relevant in the case of the truncated isoform.

Shedding of TrkB ectodomain induced by excitotoxicity has a deleterious effect on BDNF/TrkB signalling

The size of TrkB ectodomain suggests that it contains the immunoglobulin-like subdomain IgC2 [40], important for ligand interaction and specificity [41, 42]. Thus, it might be able to bind BDNF and interfere BDNF/TrkB-signalling upon excitotoxic stimulation. We induced TrkB shedding in neuronal cultures by a brief APMA treatment followed by extensive drug elimination and incubation in conditioned medium for ECD production (Figure 6A). Media collected from APMA or vehicle treated cells were added to new cultures, preincubated with TAPI-2 to avoid any secondary metalloproteinase-activation, and then treated with BDNF (50 ng/ml). A strong TrkB-FL Y515 phosphorylation (pTrkB Y515) was induced by BDNF in neurons treated with control media (Figure 6B). Interestingly, receptor-activation was greatly decreased in neurons incubated with media from APMA treated cultures, the ratio pTrkB/TrkB-FL reaching values of 39±9% compared to those of control cultures ($p<0.01$; n=3; Figure 6C). To demonstrate that interference of BDNF-signalling was solely due to TrkB ectodomain, we purified HA-ECD released by APMA treatment of HEK293T cells transfected with HA-TrkB-T1 (see supplementary material, Figure S5). Addition of purified HA-ECD to neurons significantly reduced activation of TrkB-FL induced by BDNF (25 ng/ml; Figure 6D, E), showing that this fragment is indeed an efficient BDNF-scavenger. Altogether, the experiments

above prove that TrkB ectodomain produced by metalloproteinases in excitotoxicity has a deleterious effect on BDNF/TrkB-signalling.

DISCUSSION

Stroke is a leading cause of death, dementia and disability in adults, however current therapies can only inefficiently restrict brain damage. A promising target for neuroprotection is secondary neuronal death by excitotoxicity in regions surrounding the infarct. Here, we have expanded our previous characterization of TrkB dysregulation induced by excitotoxicity and ischemia [4, 5] and its effect on BDNF/TrkB-signalling (Figure 6F). Calcium entry by NMDAR-overactivation induces pathological calpain-activation [22, 43] which acts on TrkB-FL producing a truncated membrane-anchored protein [4] and a 32-kDa cytosolic fragment. TrkB-FL calpain-processing has been recently described to occur between aminoacids N520 and S521, and contributes to neurotrophic-signalling inhibition [44]. tTrkB-FL contains Y515, an anchor of protein-adapters [45] responsible for ERK and PI3K-Akt-activation [46, 47]. Meanwhile, f32 preserves the TK domain and residue Y816 which recruits and activates PLCγ [48]. Properties of this abundant intracellular fragment suggest a cell function similarly to other TK receptors (RTKs) [36]. Thus, receptor TrkC has a TK domain highly homologous to TrkB [49], processed by caspases in the absence of neurotrophin-3 releasing a proapoptotic intracellular fragment [50]. Additionally, metalloproteinases generate a membrane-anchored TrkA fragment constitutively tyrosine-phosphorylated that can associate with signalling proteins such as Shc, p85α PI3K and PLCγ [51]. Consequently, in addition to prosurvival-signalling decrease derived from TrkB-FL degradation, both TrkB-FL calpain fragments might actively contribute to excitotoxicity.

A second calpain-independent mechanism is induced by excitotoxicity and operates *in vitro* and *in vivo* on juxtamembrane regions common to TrkB-FL and TrkB-T1 (Figure 6F), releasing a ligand binding ECD which acts as BDNF-scavenger. Similarly to receptors p75NTR [52] and TrkA [51], TrkB shedding is performed by metalloproteinases, a family of endopeptidases including matrix-metalloproteinases (MMP) and A disintegrin and metalloproteinases (ADAM) [53, 54]. MMPs are mostly secreted proteins involved in cell-growth, migration and pathologies such as ischemia [27, 28] or NDDs [26]. ADAMs are integral membrane proteins responsible for ectodomain shedding of surface proteins including growth factors, cytokines, adhesion molecules or receptors [55]. Depending on cell type or stimulus, RTKs can be processed by various metalloproteinases. Thus, phorbol esters induce p75NTR and TrkA cleavage by ADAM17 (references [56, 57]) while another unidentified metalloproteinase processes TrkA after osmotic stress [56]. N-terminal TrkB-processing by MMP-9 occurs in diabetic brain [58], a protease very important also in ischemia [59-61]. Although we cannot exclude a contribution of MMP-9 to TrkB-processing *in vivo*, results with HA-tagged TrkB receptors question a similar cleavage during *in vitro* excitotoxicity. We suggest that cleavage occurs between TrkB IgC2 and transmembrane domains, accordingly to metalloproteinases recognition of substrates stalk-regions [62]. Candidates for TrkB-cleavage are ADAM10 and ADAM17 that are responsible for most RTKs shedding [63]. Furthermore, ADAM10 is activated by NMDAR-stimulation [64] or increased intracellular-Ca^{2+} [65, 66]. Metalloproteinase-activation in excitotoxicity and ischemia additionally originates membrane-anchored CTFs f42 and f10, respectively for TrkB-FL and TrkB-T1. Similarly to other RIP substrates [25, 67], these CTFs are subsequently processed within their transmembrane regions by γ-secretases, integral membrane proteinases composed of multiple subunits. Factors governing substrate identification by γ-secretases are not well established [24], although recognition of extracellular CTF-stubs produced by previous ectodomain shedding seems

important [68]. Once released into cytosol, ICDs stability is generally low [69] although some have important functions such as Notch-ICD, which translocates to nucleus and acts as a transcription factor [70, 71]. TrkB-FL-ICD levels are very low due to efficient and functionally independent calpain-processing of f39 in excitotoxicity. For TrkB-T1-ICD, technical limitations have prevented fragment detection so we will need to further investigate its stability and putative biological function.

Our work has exposed an intricate TrkB regulation in excitotoxicity and ischemia and, therefore, it is important to establish the relative quantitative and qualitative contribution of each mechanism. Metalloproteinases play a minor role in TrkB-FL downregulation compared to calpain-cleavage, although incomplete recovery by combining both protease-inhibitors highlights the participation of additional transcriptional mechanisms [4]. Conversely, action of metalloproteinases on TrkB-T1 is very relevant in mice ischemia. Interestingly, TrkB-T1 levels are increased in human stroke [4]. As TrkB-T1 is expressed in neurons and glial cells, the discrepancy in the impact of the different mechanisms on TrkB-T1 regulation might derive from differences in glia/neuron ratios among species depending on brain complexity and size [72]. It will be interesting to establish if these proteolytic mechanisms similarly regulate TrkB in human stroke, although spontaneous calpain-activation of post-mortem brain [4] could restrict such characterization to TrkB RIP in infarcted tissue. Anyway, all TrkB regulatory mechanisms probably contribute to neuronal death regardless of their prevalence. First, TrkB-FL downregulation inhibits receptor-dependent signalling essential for neuronal survival. Secondly, neuronal TrkB-T1-processing could initially seem neuroprotective by decreasing a dominant-negative isoform, but the resulting fragments might have an active role in neurodegeneration. Thus, TrkB ectodomain maintains the ability to bind BDNF, causes aberrant BDNF/TrkB-signalling and possibly neuronal death. Actually, ECD-TrkB fused to IgG heavy chains (TrkB-Fc chimera) has been extensively used to sequester BDNF and

neurotrophin-4, reducing neurotrophic signalling [73]. Consequently, TrkB-Fc induces neuronal apoptosis [74] highlighting the importance of trophic deprivation in NDDs pathogenesis [2].

Results presented here identify TrkB isoforms as potential targets for neuroprotection and are highly relevant for the rational design of stroke therapies. Moreover, the described regulatory mechanisms might also contribute to aberrant BDNF-signalling characteristic of AD [6, 7] and other NDDs [8, 9], frequently associated to excitotoxicity [1]. In fact, amyloid-β peptide selectively increases TrkB-T1 mRNA and induces TrkB-FL calpain-cleavage [44]. Therefore, it is crucial to establish if TrkB RIP also occurs in these pathologies because BDNF-replacement, while still presenting some limitations for clinical use [2], is a promising strategy for stroke and NDDs treatment [3]. Our results strongly suggest that efficient enhancement of BDNF/TrkB-signalling will necessarily require combined BDNF and TrkB targeting.

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ABBREVIATIONS

ADAM, A disintegrin and metalloproteinase; APMA, p-Aminophenylmercuric acetate; BDNF, brain-derived neurotrophic factor; BDPs, breakdown products; Calp, calpeptin; CiIII, specific calpain-inhibitor III; CLs, cell-lysates; CM, cultured media; Comp E, Compound E; CTF, C-terminal fragment; DIVs, days *in vitro*; FL, full-length; ICD, intracellular domain; Lact, lactacystin; MMP, matrix metalloproteinase; NDDs, neurodegenerative diseases; NMDARs, NMDA type of glutamate receptors; NSE, neuronal-specific enolase; RIP, regulated intramembrane proteolysis; RTK, TK receptor; TK, tyrosine-kinase; Trk, tropomyosin-related kinase.

AUTHORS CONTRIBUTION

MD-G and GST conceived and designed the study, analysed and interpreted data, wrote the manuscript and prepared the figures. MD-G was involved in funding acquisition and group supervision. GST, RA and OGV carried out the cell culture experiments and analysed results. SA-D, GME-O and GST were involved in performing and interpreting animal experiments. All authors were involved in reviewing and commenting on the manuscript, and gave their approval of the submitted version of the manuscript.

SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Supplementary materials and methods

Figure S1. Histochemical characterization of photothrombotic focal cerebral ischemia in mice

Figure S2. Time-course of TrkB-processing depends on severity of focal cerebral ischemia

Figure S3. BDNF does not prevent TrkB-FL inactivation and metalloproteinase or calpain-processing induced by NMDAR-overactivation

Figure S4. Identification of GFP-fused CTF and ICD fragments resulting from HA-TrkB-T1-GFP RIP

Figure S5. Purification of a recombinant HA-TrkB-ECD from a heterologous system

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FIGURE LEGENDS

Figure 1. TrkB-FL-cleavage by NMDAR-overactivation results in a soluble calpain-dependent 32-kDa fragment and minor 42/39-kDa fragments. (A) Immunoblot analysis of TrkB-FL receptor in primary neuronal cultures (13 DIVs) treated with NMDA (100 µM) and glycine (10 µM) for various periods of time. Isoform-specific antibody for C-terminal regions (TrkB-FL Ct) detected endogenous TrkB full-length (FL) receptor and the resulting 32-kDa (f32) and 42/39-kDa (f42/39) cleavage fragments (upper two panels show short and long exposure times, respectively). Calpain-activation was established by analysing full-length (FL) spectrin processing into characteristic breakdown products (BDPs), and neuronal-specific enolase (NSE) was used as a loading control. (B) Graph depicts densitometry analysis of TrkB-FL and f32 band intensity, normalized to NSE levels and expressed as the percentage of the maximum value. The data shown are the means ± SEM of four independent experiments, and statistical significance was evaluated by repeated measures ANOVA and Dunnett's test (**p*<0.05, ***p*<0.01, ****p*<0.001). (C) Cultures were preincubated with proteasome-inhibitor lactacystin (20 µM) or calpain-inhibitors III (CiIII; 10 µM) and calpeptin (10 µM) before NMDA treatment for 3 h. Spectrin BDPs and levels of p53 were analysed to confirm calpain and proteasome inhibition, respectively. (D) Histogram represents quantification of normalized levels of TrkB-FL and cleavage fragments in neurons treated with NMDA, with or without protease inhibitors, as indicated in panel C. The data shown are the means ± SEM of relative protein levels (n=4) expressed as percentage of the values found in the control neurons without NMDA (TrkB-FL) or the maximum attained fragment levels (f42/39 and f32), which were all arbitrarily assigned a value of 100%. Statistical analysis was performed by ANOVA followed by Dunnett's test (**p*<0.05). (E) Double immunofluorescence detection of C-terminal region of TrkB-FL (green) together with neuronal marker synapsin (red) in untreated cultures (a) or stimulated with NMDA for 2 h (b). Confocal microscopy merged images correspond to single

sections. Boxes below show higher magnification of selected somas and dendrites. Arrowheads indicate TrkB-FL staining in plasmatic membrane. Scale bars: 10 µm.

Figure 2. TrkB-FL is also cleaved after focal cerebral ischemia in mice. (A) Immunoblot analysis of TrkB-FL in cortical infarcted regions (I) and equivalent areas of the contralateral hemisphere (C) in mice subjected to photothrombotic ischemia and sacrificed after the indicated periods of time. TrkB-FL downregulation and accumulation of its C-terminal fragments in the infarcted region correlates with calpain-activation, demonstrated by spectrin cleavage. (B and C) Histograms represent the quantification of normalized TrkB-FL and its calpain-dependent 32-kDa fragment (f32) levels (mean ± SEM, n=3) in the infarcted and contralateral regions. The data are expressed as the percentage of the value obtained in the contralateral area of sham-operated mice for TrkB-FL or the maximum value in the case of f32. Statistical significance between infarcted and contralateral regions was analysed by Student's *t*-test (*$p<0.05$, ***$p<0.001$). (D) Double immunohistochemistry with TrkB-FL C-terminal antibody (red) together with Fluoro-Jade C (FJC, green) in brain coronal sections of animals subjected to ischemic photothrombosis and sacrificed after 5 h. The ischemic region is compared to equivalent regions in the contralateral hemisphere. Arrowheads denote cell-bodies of degenerating neurons strongly stained with TrkB-FL Ct. Representative confocal microscopy images corresponding to single sections are shown. The scale bar represents 10 µm.

Figure 3. TrkB-FL is sequentially cleaved by metalloproteinases and γ-secretases in excitotoxicity. (A and B) Immunoblot analysis of excitotoxic-dependent TrkB-FL fragments in primary cortical cultures (13 DIVs) preincubated for 30 min with metalloproteinase-inhibitor TAPI-2 (10 µM), γ-secretase-inhibitor Compound E (Comp E, 1 µM) or calpain-inhibitors III (CiIII, 10 µM) and Calpeptin (Calp, 10 µM) and subsequently treated with NMDA for 6 h or left untreated. (C) Immunoblot analysis of TrkB ectodomain shedding to culture medium (CM) induced by NMDAR-overactivation. At the beginning of experimental treatments, performed as

before, conditioned medium was replaced with fresh Neurobasal medium in order to evaluate only the ectodomain (ECD) shedding produced during NMDAR-overactivation. CM was collected after treatment and analysed by immunoblot using an antibody that recognizes the N-terminal region of TrkB (panTrkB). The TrkB-FL Ct antibody was used to study the corresponding cell lysate (CL). (D and E) Time-course analysis of TrkB-FL-processing and accumulation of C-terminal intracellular fragments and TrkB ectodomain shedding in cortical cultures treated with NMDA (D) or APMA (100 μM, E) for the indicated times. (F and G) Densitometry analysis of TrkB-ECD levels in (D) and (E) (n=4, mean ± SEM) represented as fold increase compared to those obtained in non-treated cultures, arbitrarily given a value of one. Statistical significance was determined by repeated measures ANOVA with *post hoc* Dunnett's test (**p<0.01, ***p<0.001). (H) Schematic diagram of lentivirus vector LV-HA-TrkB-FL/GFP encoding proteins HA-TrkB-FL and GFP in neurons. The recombinant proteins are regulated by neurospecific synapsin promoters (pSyn) and their sequences followed by woodchuck hepatitis post-transcriptional regulatory element (WPRE). LTR, long terminal repeat. (I and J) Immunoblot analysis of LV-HA-TrkB-FL/GFP infected cultures (moi 1) incubated for 30 min with broad metalloproteinase-inhibitor GM6001 (10 μM) and subsequently treated with NMDA or APMA for 4 h. Medium was replaced at the beginning of experimental treatments with conditioned medium from non-infected cultures. Presence of soluble recombinant HA-TrkB-FL ectodomain (HA-ECD) in CM was evaluated using and antibody which recognizes the HA epitope.

Figure 4. TrkB-T1 is a substrate of RIP in neurons undergoing excitotoxicity. (A and C) Time-course analysis of TrkB-T1 receptor (T1) levels and formation of its C-terminal fragment (CTF) in cortical cultures treated with NMDA (A) or APMA (C) using an isoform-specific antibody that recognizes an intracellular region. (B) Double immunofluorescence analysis by confocal microscopy of cortical cultures untreated (a) or incubated with NMDA for 2 h (b) and

stained with TrkB-T1 (green) and synapsin (red) antibodies. Scale bars: 10 µm. (D) Graph depicts the normalized relative levels of T1 and CTF in cultures treated with APMA as before. The data shown are the means ± SEM of four independent experiments and are expressed as percentages of maximum values, which were arbitrarily assigned a 100%. Statistical significance between protein levels obtained in cultures treated with APMA relative to untreated cells was evaluated by repeated measures ANOVA (*$p<0.05$, ***$p<0.001$). (E) Schematic representation of LV-HA-TrkB-T1-GFP, which was designed for the neurospecific expression of recombinant HA-TrkB-T1 fused to GFP at its C-terminus. pSyn, neurospecific synapsin promoter; WPRE, woodchuck hepatitis post-transcriptional regulatory element; LTR, long terminal repeat. (F, G and H) Immunoblot analysis of cortical cultures infected with LV-HA-TrkB-T1-GFP and preincubated with GM6001 (10 µM; F and H) or Compound E (Comp E, 1 µM; G) for 30 min before NMDA treatment for the indicated times (F and G) or APMA addition for 6 h (H). Medium was replaced with conditioned medium from non-infected cultures immediately before drug treatments. Recombinant TrkB-T1 (HA-T1-GFP) and its C-terminal fragment (CTF-GFP) and ICD (ICD-GFP) were detected with an anti-GFP antibody. CM, culture medium. CL, cell lysate.

Figure 5. Metalloproteinases cleave TrkB-T1 and TrkB-FL after focal cerebral ischemia in mice. (A) Immunoblot analysis of TrkB-T1 (T1) cleavage and the formation of a C-terminal fragment (CTF) in mice subjected to photothrombotic ischemia and sacrificed after the indicated periods of time. Cortical infarcted regions (I) and the corresponding areas of the contralateral hemisphere (C) are compared. (B and C) Graphs depict the quantification of normalized TrkB-T1 and TrkB-T1-CTF levels (mean ± SEM, n=3) in the infarcted and contralateral regions. The data are expressed as the percentage of the value obtained in the contralateral area of sham-operated mice for TrkB-T1 or the maximum value for TrkB-T1-CTF. Statistical significance between infarcted and contralateral regions was analysed by

Student's *t*-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (D) Double immunohistochemistry with TrkB-T1 C-terminal antibody (red) together with Fluoro-Jade C (FJC, green) in brain coronal sections of animals subjected to ischemic photothrombosis and sacrificed after 5 h. The ischemic region is compared to equivalent regions in the contralateral hemisphere. Confocal microscopy images correspond to single sections and scale bar represents 10 µm. (E) Experimental timeline. GM6001 (100 mg/kg) or vehicle were injected intraperitoneally and Rose Bengal intravenously. (F) Immunoblot analysis of TrkB-FL, TrkB-T1 and their proteolytic fragments in the infarcted (I) and contralateral (C) regions from representative mice injected with GM6001 or vehicle followed by photothrombotic ischemia as depicted in (E). (G-J) Densitometry analysis of normalized levels of TrkB-FL (G), f42/39 fragments (H), TrkB-T1 (I) and TrkB-T1-CTF (J) is represented (n=4/group, mean ± SEM). Statistical significance was analysed by Student's *t*-test (*$p<0.05$, ***$p<0.001$). n.s., non-significant.

Figure 6. Purified TrkB ectodomain produced by metalloproteinases interferes BDNF-signalling. (A) Schematic representation of experiments to analyse interference of BDNF-signalling by media collected from APMA treated cells. Neuronal cultures were first incubated with vehicle or APMA (100 µM) to activate latent metalloproteinases for 1 h. After extensive drug elimination, same cultures were incubated in conditioned medium to accumulate soluble TrkB ectodomain in the absence of APMA for 4 h. This medium was transferred to new cultures preincubated with TAPI-2 to avoid any possible secondary metalloproteinase-activation, followed by treatment with BDNF (50 ng/ml) for 1 h. (B) Immunoblot analysis of TrkB-FL-activation in cultures subjected to the experimental design depicted in (A). Activation of TrkB-FL was detected using an antibody recognizing phosphorylated Y515 (pTrkB Y515) which triggers ERK and PI3K/Akt-signalling pathways. (C) Quantification of pTrkB Y515 after normalizing to total TrkB-FL and NSE in BDNF treated cultures as explained in (A) (n=3, mean ± SEM). Statistical significance was analysed by Student's *t*-test (**$p<0.01$). (D)

Experimental design of TrkB ectodomain purification and analysis of its BDNF-signalling interference in neurons. HEK293T cells transfected for 24 h for HA-TrkB-T1 expression were treated with APMA (100 μM) for 6 h. HA-TrkB ectodomain (HA-ECD) released to media was purified by immunoprecipitation and added to neurons with BDNF (25 ng/ml) for 1 h. (E) Immunoblot analysis of neurons incubated with purified HA-ECD or vehicle and BDNF as explained in (D). TrkB-FL-activation was evaluated in cell lysate (CL) as before. Corresponding culture medium (CM) was also collected at the end of the experiment to confirm addition of purified HA-ECD to neurons. (F) Histogram depicts normalized pTrkB/TrkB-FL ratio of BDNF treated neurons incubated with purified HA-ECD or vehicle (n=3, mean ± SEM). Statistical analysis was calculated using Student's *t*-test (*$p<0.05$). (G) Model of TrkB-FL/TrkB-T1 dysregulation during excitotoxicity and ischemia by three mechanisms. First one (1) consists in inversion of the physiological isoforms-mRNA ratio that favours expression of TrkB-T1 over TrkB-FL. A second (2) and critical mechanism involves TrkB-FL calpain-processing and generates a truncated receptor (tTrkB-FL) and an intracellular fragment (f32). In this work we unveil a third regulatory mechanism consisting of TrkB-FL and TrkB-T1 cleavage by RIP, which involves TrkB ectodomain shedding by metalloproteinases (3a) generating a soluble fragment (ECD) that acts as a BDNF-scavenger. The remaining C-terminal fragments (f42 for TrkB-FL and T1-CTF for TrkB-T1) are subsequently cleaved by γ-secretases (3b) releasing an intracellular domain (f39 for TrkB-FL and T1-ICD for TrkB-T1) to cytosol. The former can be further processed by calpain whereas T1-ICD might show a pathological function. All these changes alter BDNF/TrkB-signalling and contribute to neuronal excitotoxic death in stroke.

Figure S1. Histochemical characterization of photothrombotic focal cerebral ischemia in mice. Five (A) and 24 h (C) after photothrombotic brain injury, vital-dye TTC staining of coronal sections (1 mm) reliably delineate the infarct (pale areas versus non-injured deep red

coloured tissue). Nissl staining of cryosections (30 μm) prepared from 5 (B) or 24 h (D) post-injury mice brain revealed an evolving hypochromatic area in the ipsilateral neocortex (shown in detail in panels 3 and 6) indicative of neuronal injury, compared with the equivalent regions of the contralateral hemisphere (panels 1 and 4). Transition zones between damaged and non-ischemic tissue (panels 2 and 5) highlight cell morphology alterations due to ischemic injury.

Figure S2. Time-course of TrkB-processing depends on severity of focal cerebral ischemia. (A) Immunoblot analysis of TrkB-FL-processing in infarcted (I) and equivalent contralateral (C) regions of mice showing lower susceptibility to brain ischemia (CD-1 strain) subjected to photothrombosis as before and sacrificed after the indicated times (B) Quantification of normalized TrkB-FL levels. Results are represented relative to the data obtained for the contralateral region in sham-operated animals, arbitrarily assigned a 100% value (n=3, mean ± SEM). Statistical significance was evaluated by the Student's *t*-test (*$p<0.05$).

Figure S3. BDNF does not prevent TrkB-FL inactivation and metalloproteinase or calpain-processing induced by NMDAR-overactivation. Cortical cultures incubated with BDNF (100 ng/ml) and/or NMDA for the indicated periods of time were analysed by immunoblot.

Figure S4. Identification of GFP-fused CTF and ICD fragments resulting from HA-TrkB-T1-GFP RIP. Immunoblot analysis of cortical cultures infected with LV-HA-TrkB-T1-GFP (moi 1) and treated with NMDA for 6 h compared to cells infected with the same multiplicity of control virus LV-GFP. Recombinant TrkB-T1 C-terminal fragment (CTF-GFP) and intracellular domain (ICD-GFP) were identified with an anti-GFP antibody and different exposure times of the original blot are shown for each lentivirus.

Figure S5. Purification of a recombinant HA-TrkB-ECD from a heterologous system. HEK293T cells transfected with plasmid HA-TrkB-T1 were treated with APMA (100 μM) for 6 h before media collection. Released HA-ECD fragment was then purified by using anti-HA conjugated agarose beads. Immunoblot with HA-antibodies of collected medium and first three eluted fractions is shown.