Excitotoxicity-induced endocytosis as a potential target for stroke neuroprotection

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Decreased neuronal survival-signaling and brain damage: Stroke is a leading cause of death worldwide, the major cause of adult disability and second of dementia. In spite of the social and economic importance of this disorder, and after intense research, no effective drugs have yet reached the clinic. Blood reperfusion with the thrombolytic agent tissue plasminogen activator remains the only pharmacologic treatment currently available for ischemic stroke, the major type of brain injury (e.g., cardiovascular cases). Damage in this situation results from thrombotic or embolic occlusion of a cerebral artery causing a decrease of blood flow to a specific area of the brain parenchyma, neurons being particularly sensitive to a reduction of the supply of glucose and oxygen. It is thus a predictable event in stroke that, in a physiological scenario, neurons are able to preserve neurons from the ischemic injury and, in this way, reduce brain damage and patient disability. A promising approach involves rescue of the area of penumbra surrounding the infarct, a region functionally silent but structurally intact. However, neurons in models of delayed cell death, known as a process of delayed death known as excitotoxicity, caused by overstimulation of the N-methyl-D-aspartate receptor. The critical role played by these receptors in synaptic plasticity, learning and memory, together with dual functions in neuronal survival and death (Hardingham et al., 2002), underlies previous failure of NMDAR blockade as a therapeutic target in stroke. Nevertheless, the low-affinity competitive NMDAR antagonist memantine is still able to improve cognitive functions and behavioral disturbances in moderate-to-severe Alzheimer’s disease, a neurodegenerative disorder also associated with excitotoxicity. Anyhow, for stroke treatment, we are currently exploring alternative strategies such as the inhibition of neurotoxic proteins that act downstream overactivated NMDARs or directed to enhance neuronal survival pathways. Concerning the latter, several laboratories have chosen to analyze the modulation of neurotransmitter-dependent survival pathways by treatment with brain-derived neurotrophic factor (BDNF) as a possible strategy for neuroprotection in stroke but also other acute or chronic disorders of the central nervous system. However, a potential caveat of this approach is that signaling mediated by BDNF is dramatically altered by excitotoxicity, a process not only central to stroke but, as mentioned, also associated to many other neurological disorders (Tejeda and Diaz-Guerra, 2017). In models of stroke and human samples, excitotoxicity induces transcriptional and proteolytic mechanisms strongly associated with neurodegeneration that alter the expression of the two major brain isoforms of the BDNF receptor, tropomyosin-related kinase B (TrkB); the catalytically active full-length receptor (TrkB-FL) and a truncated receptor lacking the tyrosine kinase domain (TrkB-T1) (Vidaurre et al., 2012; Tejeda et al., 2016). Nonetheless, recent work from my group has demonstrated that it is possible to interfere TrkB-FL degradation in stroke and, in this way, decrease neuronal death and brain damage (Tejeda et al., 2019). Interestingly, these results have been accomplished by primarily preventing TrkB-FL endocytosis, which is strongly induced by excitotoxicity and precedes neuronal death (Figure 1). In this perspective, we will discuss the prospects of using the modulation of excitotoxicity-induced endocytosis, and the subsequent preservation of membrane survival proteins, as a neuroprotective therapeutic strategy for acute brain insults (stroke, epilepsy, or trauma), and excitotoxicity-associated chronic disorders (e.g., Alzheimer’s, Parkinson’s, Huntington’s diseases).

Dual role of endocytosis in neuronal survival and death: Endocytosis is a ubiquitous physiological process that mediates nutrient uptake, receptor internalization and signaling, essential events for cell growth and survival. In neurons, endocytosis is required at the synaptic cleft after neurotransmitter release for recycling of membranes and surface proteins. Meanwhile, in the postsynaptic membranes of glutamnergic neurons, endocytosis is central for the reduction in long-term depression of surface glutamate receptors, mostly of the a-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid family. NMDARs also undergo internalization in response to ligand binding, synapse maturation or long-term depression. Consequences of GluN2A/B subunits direct endocytosed NMDARs to recycling endosomes, conserved motifs near the juxtamembrane region of GluN1 and GluN2A/B drive receptors to late endosomes and degradation (Scott et al., 2004). Notably, trafficking of TrkB isoforms is similarly very important for neurotrophin signaling in physiological conditions. Upon BDNF binding, both isoforms are rapidly and efficiently internalized in a clathrin-dependent way and form signaling endosomes. However, while TrkB-T1 predominantly recycles back to the cell surface by a default mechanism, TrkB-FL recycling is less efficient, relies on its tyrosine kinase activity, and is regulated by binding of the protein Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) to a receptor juxtamembrane region located between the transmembrane and tyrosine kinase domains (Huang et al., 2009).

In addition, endocytosis plays an evolutionarily conserved function in cell destruction by neurotoxicity, a formidable cellular insult to stroke and other diseases affecting the central nervous system (Troulinaki and Tavernarakis, 2012). A transient upregulation of the endocytic activity has been observed early after cell death induction which is related to changes in calcium homeostasis taking place along neurodegeneration. The NMDARs are efficient calcium channels and their overactivation, among other mechanisms, profoundly alters the intracellular redox balance. Correspondingly, endocytosis is enhanced in excitotoxicity by a clathrin/dynamin-mediated mechanism that precedes neuronal death in vitro or in models of ischemia, where stressed but viable neurons of the ischemic region have been described as highly endocytic (Vaslin et al., 2009). A crosstalk between endocytosis and the activation by Ca2+ of the protease calpain has been also described. After NMDAR overstimulation, cleavage by calpain of different substrates triggers multiple mechanisms of neurotoxicity, particularly enhancement of calcium load, disruption of cell structure and promotion of cell-death signaling by degradation of antiapoptotic proteins or, alternatively, those involved in neuroprotective signaling such as TrkB-FL (Vidaurre et al., 2012). The subcellular localization of calpain activation is currently under discussion. Activation is favored near plasma and endosomal membranes but can also occur in microdomains with high local [Ca2+] as well as other cell compartments (mitochondria, nucleus or Golgi membranes). Notably, several components of the clathrin-coated vesicles, including the a- and b2-subunits of the adaptor protein complex 2 (AP-2), are calpain substrates and result hydrolyzed in experimental ischemia and the brain of Alzheimer’s disease patients (Rudinskly et al., 2009). Cleavage of these AP-2 proteins has been suggested to be a mechanism to reduce clathrin-coated vesicles and moderate endocytosis at late stages of necrotic cell death.

Prevention of TrkB-FL endocytosis is relevant for stroke neuroprotection in vivo: Since endocytosis is a requirement for necrosis, general strategies to downregulate or deplete key proteins mediating different steps of the endocytic pathway have been devised and shown to actually interfere with neurodegeneration (Troulinaki and Tavernarakis, 2012). Therefore, generic interference of endocytosis might be considered as a therapeutic strategy with potential to reduce cell death due to acute brain insults. However, potential caveats to this approach are the pleiotropic adverse effects it might have considering that essential physiological cell processes would be inhibited as well. As an alternative, we propose the use of molecules able to interfere pathological endocytosis of specific neuronal survival proteins. One of these strategies is the NMDAR, internalized by its own overstimulation. In fact, endocytosis of GluN2B-containing NMDARs has been shown to mediate excitotoxicity by still unknown mechanisms (Wu et al., 2017). Blockade of this clathrin-dependent endocytic process using a cell-penetrating peptide (CPP) was able to inhibit excitotoxicity in cultured cortical neurons. This CPP (Tat-YEKL) contains a 11 aa domain of the human immunodeficiency virus type 1 Tat protein, which allows attached cargoes to cross the blood-brain barrier and plasma membrane, followed by a 12 aa of CPP corresponding to the AP-2 binding motif. The selection of CPPs as neuroprotective tools for stroke therapy seems particularly appropriate.
since the highly endocytic neurons of the ischemic area present an enhanced uptake of Tat-derived peptides (Vaslin et al., 2009) which results in a selective targeting of potential neuroprotective CPPs to damaged neurons. In the future, it will be interesting to establish if this CPP has similar neuroprotective effects when used in vivo and can specifically inhibit pro-death effects of NMDAR overactivation while preserving other receptor functions. Another possible candidate to be explored as neuroprotective target is kinase D-interacting substrate of 220 kDa (Kidins220), a downstream effector of neurotrophin receptors and NMDARs essential for neuronal viability. We have demonstrated that Kidins220 processing by calpain in excitotoxicity is secondary to traffic of this protein to the Golgi apparatus and early activation of Rap1-GTPase (López-Menéndez et al., 2019). At later stages of the excitotoxic process, Kidins220 downregulation governs Rap1 inactivation associated to a decrease in ERK activity which compromises neuronal survival. Therefore, prevention of excitotoxicity-induced Kidins220 endocytosis might be investigated as a way to inhibit calpain processing of Rap1 activation complexes and, thus, interfere the shut-off of Kidins220/Rap1/ERK prosurvival cascades.

Finally, we have already demonstrated that interference of TrkB-FL endocytosis is a relevant neuroprotective strategy both in vitro and in vivo. We have found that excitotoxicity decreases the surface levels of this receptor and, sequentially, promotes its intracellular processing by several proteases (Tejeda et al., 2019). The major mechanism of TrKB-FL downregulation is cleavage of its intracellular juxtamembrane region by calpain (Viduaurre et al., 2012; Figure 1). A secondary mechanism for TrkB-FL, but primary for TrkB-T1 regulation, is regulated intramembrane proteolysis by sequential metalloproteinase/γ-secretase action, producing the shedding by these isoforms of identical receptor ectodomains that we have demonstrated act as BDNF-scavengers (Tejeda et al., 2016). Altogether, these mechanisms cause a profound alteration of BDNF signaling by excitotoxicity, observed not only in vitro but also other neurological disorders (Tejeda and Diaz-Guerra, 2017). In order to preserve BDNF-regulated survival pathways, we have designed a Tat-derived CPP (TFL457) containing a short TrkB-FL juxtamembrane sequence (aa 457–471) that we hypothesized might be important for the control of receptor stability and function in excitotoxicity. The selected sequence is part of an intracellular region established as important for regulation of TrkB-FL location and function through interaction with a set of different proteins that include Hrs (Huang et al., 2009). We observed that peptide TFL457, specifically prevented early TrkB-FL endocytosis activation by excitotoxicity, differently from a negative control CPP containing non-related sequences (TMyc). We hypothesized that TFL457 might be competing some protein/s interaction/s established by sequence 457–471 in TrkB-FL which would be required for the promotion of excitotoxicity-induced endocytosis. Since this peptide will only affect interactions specifically established by TrkB-FL, endocytosis of other surface proteins would not be affected. By keeping the unprocessed receptor in the cell surface, TFL457 secondarily interferes

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**Figure 1 | Mechanism of action of neuroprotective peptide TFL457.** The CPP contains a TrkB-FL juxtamembrane sequence (dark green rectangle) that probably competes a TrkB-interacting protein (orange) important for excitotoxicity-induced endocytosis, thus preventing secondary receptor processing and neuronal death. The interaction of TrkB with this unmodified protein is maintained in the presence of the control peptide TMyc (purple circle) and neuronal death is induced by the excitotoxic insult. CPP: Cell-penetrating peptide; TFL457 and TMyc: Tat-derived CPPs; TrkB: tropomisin-related kinase B; TrkB-FL: the catalytically active full-length receptor.