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Calpain-dependent truncated form of TrkB-FL increases in neurodegenerative processes

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ABSTRACT

Recent findings indicate that the mechanisms that drive reshaping of the nervous system are aberrantly activated in epilepsy and several neurodegenerative diseases. The recurrent seizures in epilepsy, particularly in the condition called *status epilepticus*, can cause permanent neurological damage, resulting in cognitive dysfunction and other serious neurological conditions. In this study, we used an in vitro model of *status epilepticus* to examine the role of calpain in the degeneration of hippocampal neurons. We grew neurons on a culture system that allowed studying the dendritic and axonal domains separately from the cell bodies. We found that a recently characterized calpain substrate, the neurotrophin receptor TrkB, is cleaved in the dendritic and axonal domain of neurons committed to die, and this constitutes an early step in the neuronal degeneration process. While the full-length TrkB (TrkB-FL) levels decreased, the truncated form of TrkB (Tc TrkB-FL) concurrently increased, leading to a TrkB-FL/Tc TrkB-FL imbalance, which is thought to be causally linked to neurodegeneration. We further show that the treatment with *N*-acetyl-Leu-Leu-norleucinal, a specific calpain activity blocker, fully protects the neuronal processes from degeneration, prevents the TrkB-FL/Tc TrkB-FL imbalance, and provides full neuroprotection. Moreover, the use of the TrkB antagonist ANA 12 at the time when the levels of TrkB-FL were significantly decreased, totally blocked neuronal death, suggesting that Tc TrkB-FL may have a role in neuronal death. These results indicate that the imbalance of these neurotrophins receptors plays a key role in neurite degeneration induced by seizures.

1. Introduction

Most neurons initially develop an extensive set of neuritic processes that are subsequently pruned, so that only those that are part of the mature neuronal network are retained. In addition, significant sculpting of neuritic processes can occur throughout the neuron's lifespan (Pease and Segal, 2014). The changes in the morphological structure of neurites are mediated by conserved signaling mechanisms that are now beginning to be understood. Importantly, recent findings indicate that the mechanisms driving the reshaping of the nervous system are aberrantly activated in neurodegenerative diseases (Liu et al., 2008; Ma, 2013). The term "neurodegeneration" is defined as the progressive loss of structure and function of neurons, which ultimately leads to neuronal death (Yildiz-Unal et al., 2015).

Epilepsy is a disease of the central nervous system (CNS) characterized by recurrent seizures, and affects 1–2% of the population worldwide (Harvey and Sloviter, 2005). A seizure is a clinical manifestation of abnormal, disordered and synchronized high-frequency firing of neuronal populations of the CNS (De Lorenzo et al., 2005). In some circumstances, the seizures do not stop spontaneously. If their duration exceeds 30 min they are classified as *status epilepticus* (SE), which can cause permanent neurological damage, resulting in cognitive dysfunction and other serious neurological deficits (Liu et al., 2013).

The neuronal populations engaged in SE characteristically show an increase in intracellular calcium levels ($[Ca^{2+}]^i$) (DeLorenzo et al., 2005) resulting from massive calcium entry. It has been demonstrated that this calcium inflow induces changes ranging from an increase in the expression levels of several genes to the activation of proteases (Hardingham and Bading, 2010), calpains among them. Calpains are a family of Ca^{2+}-dependent non-lysosomal cysteine proteases (Perlmutter et al., 1990; Goll et al., 2003; Ma, 2013), whose substrates include cytoskeletal proteins (i.e. α-spectrin, neurofilaments), membrane receptors (e.g. the NMDA receptor) (Gladding et al., 2012), the neurotrophin receptor TrkB (Vidaurre et al., 2012; Gomes et al., 2012;

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Xie et al., 2014; Jerónimo-Santos et al., 2015), and other proteases, such as caspases (Blomgren et al., 2001; Graham et al., 2006). The cleavage products of calpains action on their substrate may show biological activity (Ma, 2013).

One of the most distinctive features of SE is that it induces an increase in the levels of BDNF and proBDNF, as well as an imbalance in the expression of their receptors, TrkB and p75ntr, respectively, before the onset of neuronal death, a feature shared with other neurodegenerative diseases (Unsain et al., 2008, 2009; Brito et al., 2013). In this regard, it has been demonstrated in models of ischemia, Alzheimer disease (AD) and SE, that calpain can process TrkB full-length (TrkB-FL), producing a cleaved receptor, the truncated TrkB-FL (Tc TrkB-FL), which is similar to the splice-variant TrkB T1 in that both receptors lack most of the intracellular domain. The biological consequence of this processing is still a matter of debate (Vidaurre et al., 2012; Gomes et al., 2012; Xie et al., 2014; Jerónimo-Santos et al., 2015). Because neurite degeneration is a common characteristic of these conditions, it is possible that TrkB-FL decreased, and Tc TrkB-FL increased initially in dendritic and axonal domains of neurons committed to die.

Our results show that the rise in intracellular calcium levels during SE activates calpain, which in turn processes TrkB receptors in dendrites and axons, and suggest that this phenomenon contributes to neuronal degeneration during SE in hippocampal neurons.

2. Materials and methods

2.1. Primary neuronal cultures

Cultures of rat hippocampal neurons were prepared from E17–18 Wistar embryos as previously described (Kaech and Banker, 2006), with minor modifications. Cells were cultured at a density of 50,000 cells/cm^2 on poly-L-lysine-coated coverslips or on cell culture filter inserts (1 μm pore size, BD Falcon) (for immunocytochemistry) and on Corning plate dishes or on cell culture filter inserts (1 μm pore size, BD Falcon) (for Western Blot, WB). Hippocampal cultures were kept in maintenance medium composed of Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), glutamine (0.5 mM), and Pen/Strep 0.5%. The neurons were kept at 37 °C in a humidified incubator with 5% CO_2/95% air for 10–11 days. After the second day in vitro (DIV), AraC or FDU was added to the medium to prevent glial cell proliferation (at 10 DIV, GFAP-positive cells represented about 5% of the cell population, data not shown). The inhibitor calpain ALLN (Calbiochem) and ANA12 (Cazorla et al., 2011) were added at 10uM in the indicated experiments. The animals used in the preparation of cell cultures were handled according to the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. In vitro model of SE protocol

The SE protocol consisted of exposing the culture to a $MgCl_2$-free buffer solution (Sombati and Delorenzo, 1995). On the 10/11th DIV, the maintenance medium was replaced by control or $MgCl_2$-free buffer solution containing (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 10 glucose, 0.002 glycine, plus or minus 1 $MgCl_2$, pH 7.3. Three washes (3×1.5 ml of buffer solution) were performed to remove all traces of Neurobasal medium. The cells were then incubated in the appropriate buffer solution at 37 °C in an atmosphere of 5% CO_2/95% air for 3 h. The neurons were incubated again in maintenance medium under the same temperature and atmosphere conditions. We analyzed and quantified the data at the following time points after the end of the SE stimulus: 0 (immediately after), 6, and 12 h for assessing cell survival over time and the pattern of neurodegeneration, and 0, 3 and 6 h for the WB experiments.

2.3. Validation of the in vitro model of SE: calcium images

Fluo4-AM, a well-characterized calcium fluorescent marker, was used to quantify the intracellular concentration of this ion (Gee et al., 2000). On the 10th DIV, the maintenance medium was replaced with control buffer (i.e. with Mg^{2+}), and Fluo4-AM (5 mM) was subsequently added to "load" the neurons with the dye. The neurons were incubated for 30 min in the presence of the calcium dye, followed by two washes with control buffer to remove the excess of Fluo4-AM. Following this, the cells were filmed during 35 min. The test was repeated three times. We used the following formula to quantify the average concentration of intracellular calcium during the SE in vitro: Calcium concentration = (fluorescence intensity of calcium / calcium average intensity over the basal time period of 5 min) $\times$ 100.

2.4. Neuronal death assay

Neuronal death was evaluated by two different methods: calcein/ethidium staining, and assessing the presence/absence of the nuclear protein NeuN (Liu et al., 2013). To perform these assays, six random fields in each coverslip were selected for analysis. The number of calcein-positive neurons was divided by the number of those labeled with ethidium and calcein (total of neurons), taking the control as 100%. The percentage of neuronal death was calculated as the number of neurons labeled with NeuN, with respect to number of labeled neurons in the control condition, taken as 100%. At least two coverslips with cultured neurons were evaluated for each condition, and each experiment was repeated three times.

2.5. Immunocytochemistry

Hippocampal neurons grown on cell culture filter inserts (1 μm pore size, BD Falcon) or bulk cultures were fixed with 4% paraformaldehyde for 10 min at room temperature, and then blocked with blocking solution containing TBS-T, 5% skim milk and 0.3% Triton X-100. Hippocampal neurons were then incubated overnight at 4 °C with antibodies against β-III tubulin (1:20,000, MAB 5564), MAP2 antibody (Sigma M1406, diluted 1:10,000 in blocking solution), TrkB 8316 Santa Cruz (1/750), pTrkB-Y817 rabbit Epitomics 2149-1 (1/1000), NeuN (1:500, MAB 377) and NF-M (1:1000, Millipore AB 1987). Filter inserts or bulk cultures were subsequently incubated with Alexa 488- or 555-conjugated goat anti-mouse/rabbit secondary antibodies for 1 h at room temperature. The filters were removed from the insert, placed in fluorescent mounting medium, and mounted on Superfrost Plus slides (Fisher Scientific).

2.6. Western blot

Hippocampal neuronal cultures were washed twice with ice-cold PBS, followed by a wash with PBS buffer. The cells were then lysed with RIPA buffer supplemented with the mixture of protease and phosphatase inhibitors. After centrifugation at 16,000 × *g* for 10 min, proteins in the supernatants were quantified, and the samples were denatured with concentrated Laemmli denaturing buffer at 95 °C for 5 min. Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels (α-spectrin, TrkB, pTrkB, NF-M and p75ntr) and in 15% (procaspase-3), transferred to nitrocellulose membranes (Millipore). The blots were incubated with primary antibodies (overnight at 4 °C), washed, and reincubated with the secondary HRP conjugated antibody (1:2000 dilution for anti-rabbit and anti-mouse IgG; 1 h at room temperature). Peroxidase activity was visualized by enhanced chemiluminescence on the ECL and blot imaging system, and quantified with the FIJI program (From NIH). The following primary antibodies were utilized: anti-TrkB (1:750, Milipore 07-225), anti-spectrin (1:1000, MAB1622; Millipore Bioscience Research Reagents), pTrkB-Y817 rabbit Epitomics 2149-1 (1/1000), Rex antibody (1:1000), Anti NF-M (1:1000, Millipore AB