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Cleavage of the vesicular glutamate transporters under excitotoxic conditions

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ABSTRACT

Glutamate is loaded into synaptic vesicles by vesicular glutamate transporters (VGLUTs), and alterations in the transporters expression directly regulate neurotransmitter release. We investigated changes in VGLUT1 and VGLUT2 protein levels after ischemic and excitotoxic insults. The results show that VGLUT2 is cleaved by calpains after excitotoxic stimulation of hippocampal neurons with glutamate, whereas VGLUT1 is downregulated to a lower extent. VGLUT2 was also cleaved by calpains after oxygen/glucose deprivation (OGD), and downregulated after middle cerebral artery occlusion (MCAO) and intrahippocampal injection of kainate. In contrast, VGLUT1 was not affected after OGD. Incubation of isolated synaptic vesicles with recombinant calpain also induced VGLUT2 cleavage, with a little effect observed for VGLUT1. N-terminal sequencing analysis showed that calpain cleaves VGLUT2 in the C-terminus, at Asn^{534} and Lys^{542}. The truncated GFP-VGLUT2 forms were found to a great extent in non-synaptic regions along neurites, when compared to GFP-VGLUT2. These findings show that excitotoxic and ischemic insults downregulate VGLUT2, which is likely to affect glutamatergic transmission and cell death, especially in the neonatal period when the transporter is expressed at higher levels.

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Introduction

Glutamate plays a critical role in the pathophysiology of cerebral ischemia (Doyle et al., 2008). Excitotoxicity caused by overactivation of glutamate receptors contributes to neuronal death in several neuronal pathologies, including ischemia, epilepsy and neurodegenerative disorders (Szydlowska and Tymianski, 2010). The $[Ca^{2+}]_i$ overload resulting from the overactivation of glutamate receptors induces an excessive stimulation of calpains, a family of cysteine proteases activated by calcium, leading to the cleavage of several substrates (Bevers and Neumar, 2008). These include proteins that play key roles in glutamatergic synapses, including AMPA (Yuen et al., 2007) and NMDA receptor subunits (Gascon et al., 2008), and mGluR1 metabotropic glutamate receptors (Xu et al., 2007). Caspase activation also contributes to neuronal death in brain ischemia (Broughton et al., 2009), and these proteases mediate the degradation of AMPA receptor subunits during early periods of cell stress. The resulting downregulation of excitatory activity may ensure apoptosis by preventing excitotoxic necrosis (Glazner et al., 2000).

The activity of glutamatergic synapses is regulated by plasma membrane and vesicular glutamate transporters. The reversal of plasma membrane-associated glutamate transporters is thought to contribute to excitotoxic neuronal damage in brain ischemia (Chao et al., 2010). Changes in the abundance of vesicular glutamate transporters (VGLUT) were also reported in brain ischemia. VGLUT2 is downregulated in the CA1 layer of the gerbil hippocampus after a mild transient period of global ischemia, and this change was associated with delayed neuronal death (Kim et al., 2006). Similarly, VGLUT2 and VGLUT3 were also found to be downregulated in the cerebral cortex and caudate–putamen of rats subjected to transient MCAO. In contrast, VGLUT1 protein levels were transiently increased in the same brain regions during the first 3 days of reperfusion, but decreased 7 days after the ischemic insult (Sanchez-Mendoza et al., 2010). VGLUT1 and VGLUT2 account for the ability of most excitatory neurons to release glutamate by exocytosis (Fremeau et al., 2004). VGLUT1 levels increase gradually after birth, becoming the dominant form in the adult brain, whereas VGLUT2 shows a higher expression early in development (Boulland et al., 2004). Regulation of VGLUT expression may affect quantal size (Wojcik et al., 2004) and, therefore,

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changes in their expression may affect glutamate release in brain ischemia, particularly in the penumbra region where ATP is available to allow exocytosis to occur.

In this work we further investigated the effects of excitotoxic stimulation and *in vivo* and *in vitro* brain ischemia in the vesicular glutamate transporter protein levels. The results show that VGLUT2 is particularly sensitive to excitotoxic stimulation, and the cleavage of the transporter by calpains gives rise to a truncated form that is not targeted to synapses.

Material and methods

DNA constructs

To clone the VGLUT2 C-terminal in pGEX6P2, the cDNA encoding the C-terminal of rat (*Rattus norvegicus*) VGLUT2 was obtained by RT-PCR from rat cerebral cortex RNA. The first strand cDNA was produced using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche), and the resulting cDNA was amplified by PCR using primers forward 5′-CGGAATTCTATTTGCCTCAGGAGAGAAG-3′ and reverse 5′-CCGCTCGAGT-TATGAATAATCATCTCGGT-3′. This product was cloned into pGEM vector (Promega), and then subcloned into pGEX6P2 using EcoRI and XhoI restriction sites, in frame with glutathione S-transferase (GST). The pGEX-VGLUT2 C-terminus sequence was confirmed by DNA sequencing reactions. The plasmids encoding for the fusion proteins of GST-VGLUT2 C-terminal mutants, D522A and D525A, were produced by site-directed mutagenesis, using PCR.

The rat VGLUT2 in pCMV6b was a kind gift from J. Takeda (Gifu University, Japan). The plasmid encoding VGLUT2 rat protein fused with green fluorescent protein (GFP) was generated by molecular cloning. The cDNA of rat VGLUT2 was amplified by PCR using the primers forward 5′-CGGAATTCTATGGAGTCGGTAAAACAAAG-3′ and reverse 5′-CGGGATCCTTATGAATAATCATCTCGG-3′, and subcloned into pGEM vector (Promega) through EcoRI and BamHI restriction sites. VGLUT2 was then subcloned into pEGFPC1 (BD Biosciences Clontech) in frame with GFP. The pEGFPC1-VGLUT2 sequence was verified by DNA sequencing reactions. The truncated pGFPC1-VGLUT2 plasmids were generated by insertion of stop codons after amino acids 533 or 541 by directed mutagenesis, using PCR.

The rat VGLUT1 in pcDNA3.1(+) at HindIII/XhoI site was a kind gift of S. Takamori (Tokyo University, Japan). The plasmid encoding the GST-VGLUT1 C-terminus fusion protein was produced by PCR using the primers forward 5′-CCGGAATTCTCGGGAGAGAAACAGCCGTGG-3′ and reverse 5′-CCGCTCGAGTCAGTAGTCCCGGACAGGGGG-3′ to amplify VGLUT1 C-terminus. The PCR product was cloned into pGEX4T1 vector (Pharmacia) with EcoRI and XhoI. The pGEX4T1-VGLUT1 C-terminus sequence was confirmed by DNA sequencing reactions.

Hippocampal cultures

High density cultures of rat hippocampal neurons were prepared from E18–E19 Wistar rat embryos as previously described (Gomes et al., 2011). Neuronal cultures were maintained in serum-free Neurobasal medium (Gibco Invitrogen), supplemented with B27 (Gibco Invitrogen), glutamate (25 μM), glutamine (0.5 mM) and gentamycin (0.12 mg/ml). Cells were cultured at a density of 9×10^4 cells/cm^2 on poly-D-lysine coated 6-well microplates (MW6), or at 2×10^5 cells/cm^2 on poly-D-lysine coated coverslips (10 mm), and kept at 37 °C in a humidified incubator with 5% CO_2/95% air, for 7 or 15 days *in vitro* (DIV).

Low density hippocampal cultures were prepared as previously described (Goslin et al., 1998). Briefly, hippocampi were dissected from E18 rat embryos and dissociated using trypsin (0.25%). Neurons were plated at a final density of $1–5\times10^4$ cells/dish (60 mm culture dishes) on poly-D-lysine-coated coverslips and cultured in the presence of an astroglial feeder layer. Cultures were maintained in Neurobasal medium supplemented with B27 supplement (1:50), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. To prevent the overgrowth of the glia, neuron cultures were treated with 5 μM cytosine arabinoside after 3 DIV and maintained in a humidified incubator with 5% CO_2/95% air, at 37 °C, for up to 2 weeks, feeding the cells once per week by replacing one-third of the medium.

Excitotoxic stimulation with glutamate

Hippocampal neurons (7 DIV) were exposed to 125 μM glutamate for 20 min in Neurobasal medium and further incubated in culture conditioned medium for the indicated periods of time. Pre-incubations of 2 h were used when cells were treated with the calpain inhibitors MDL28170 (Calbiochem) and ALLN (Calbiochem; 50 μM), or with the pan-caspase inhibitor Z-VAD-FMK (Biomol; 50 μM). Under control conditions, neurons were not exposed to glutamate.

Induction of apoptosis

Hippocampal neurons (7 DIV) were exposed to 30 nM staurosporine for 24 h (Prehn et al., 1997), or to 300 nM for 10 min (Lankiewicz et al., 2000), and further incubated in culture conditioned medium for 24 h. Withdrawal of trophic factor support was accomplished by replacing culture medium with Locke's buffer for 48 h, as previously described (Guo et al., 2008).

Oxygen–glucose deprivation (OGD)

Hippocampal neurons (15 DIV) were incubated in a glucose-free saline buffer (116 mM NaCl, 25 mM sucrose, 10 mM Hepes, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$) under an anaerobic atmosphere (10% H_2, 85% N_2, 5% CO_2) (Forma Anaerobic System, Thermo Fisher Scientific), at 37 °C for 1.5 h. After the OGD challenge, the buffer was replaced by culture conditioned medium and the cells were returned to the humidified 5% CO_2/95% air incubator for 8 h. Control neurons (Sham) were incubated in the saline buffer described above, supplemented with 25 mM glucose (in the absence of sucrose), and kept in the humidified 5% CO_2/95% air incubator at 37 °C. Pre-incubations of 2 h were used when cells were treated with the calpain inhibitor ALLN (50 μM), and the inhibitor was also present during the post-incubation period. For nuclear morphology analysis hippocampal neurons (15 DIV) were subjected to OGD for 1.5 h followed by 12 h of post-incubation in culture conditioned medium. After fixation the cells were incubated with 1 μg/ml Hoechst 33342 for 10 min. Three coverslips were prepared for each experimental condition, and at least 200 cells were counted in each case.

Preparation of extracts

Hippocampal culture extracts were prepared as previously described (Gomes et al., 2011). Briefly, neurons were washed with ice-cold PBS, and lysed with RIPA. After centrifugation, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce), and the samples were denaturated with 2× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue). Extracts used for VGLUT1 and VGLUT2 analysis were not boiled to avoid VGLUT aggregation.

Western blot

Protein samples were separated by SDS-PAGE, in 12% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and immunoblotted as previously described (Gomes et al., 2011). Blots were incubated with primary antibodies overnight at 4 °C, washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20,000 dilution for anti-rabbit IgG and 1:10,000 dilution

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