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Multiple domains in the C-terminus of NMDA receptor GluN2B subunit contribute to neuronal death following *in vitro* ischemia

Marta M. Vieira [a], Jeannette Schmidt [a,b], Joana S. Ferreira [a], Kevin She [c], Shinichiro Oku [c], Miranda Mele [a], Armanda E. Santos [a,d], Carlos B. Duarte [a,e], Ann Marie Craig [c], Ana Luísa Carvalho [a,e,*]

[a] CNC — Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[b] PDBEB — Doctoral Program in Experimental Biology and Biomedicine, Center for Neuroscience and Cell Biology, Institute for Interdisciplinary Research, University of Coimbra, Coimbra, Portugal
[c] Brain Research Centre and Department of Psychiatry, University of British Columbia, Vancouver, BC, Canada
[d] Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal
[e] Department of Life Sciences, University of Coimbra, Coimbra, Portugal

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ABSTRACT

Global cerebral ischemia induces selective degeneration of specific subsets of neurons throughout the brain, particularly in the hippocampus and cortex. One of the major hallmarks of cerebral ischemia is excitotoxicity, characterized by overactivation of glutamate receptors leading to intracellular Ca^{2+} overload and ultimately neuronal demise. N-methyl-D-aspartate receptors (NMDARs) are considered to be largely responsible for excitotoxic injury due to their high Ca^{2+} permeability. In the hippocampus and cortex, these receptors are most prominently composed of combinations of two GluN1 subunits and two GluN2A and/or GluN2B subunits. Due to the controversy regarding the differential role of GluN2A and GluN2B subunits in excitotoxic cell death, we investigated the role of GluN2B in the activation of pro-death signaling following an *in vitro* model of global ischemia, oxygen and glucose deprivation (OGD). For this purpose, we used GluN2B$^{-/-}$ mouse cortical cultures and observed that OGD-induced damage was reduced in these neurons, and partially prevented in wild-type rat neurons by a selective GluN2B antagonist. Notably, we found a crucial role of the C-terminal domain of the GluN2B subunit in triggering excitotoxic signaling. Indeed, expression of YFP–GluN2B C-terminus mutants for the binding sites to post-synaptic density protein 95 (PSD95), Ca^{2+}-calmodulin kinase IIα (CaMKIIα) or clathrin adaptor protein 2 (AP2) failed to mediate neuronal death in OGD conditions. We focused on the GluN2B–CaMKIIα interaction and found a determinant role of this interaction in OGD-induced death. Inhibition or knock-down of CaMKIIα exerted a neuroprotective effect against OGD-induced death, whereas overexpression of this kinase had a detrimental effect. Importantly, in comparison with neurons overexpressing wild-type CaMKIIα, neurons overexpressing a mutant form of the kinase (CaMKII-I205K), unable to interact with GluN2B, were partially protected against OGD-induced damage. Taken together, our results identify crucial determinants in the C-terminal domain of GluN2B subunits in promoting neuronal death in ischemic conditions. These mechanisms underlie the divergent roles of the GluN2A- and GluN2B–NMDARs in determining neuronal fate in cerebral ischemia.

1. Introduction

Transient cerebral global ischemia is currently one of the most common causes of disability and mortality. It results from a lack of blood supply to the entire brain that may arise due to cardiac arrest, leading to the delayed death of specific subpopulations of neurons. The hippocampus and some of the cerebral cortical layers are prominently affected following global cerebral ischemia in patients (Petito et al., 1987) and also in animal models (Kirino, 1982; Zukin et al., 2004). Global cerebral ischemic insults can be reproduced *in vitro* by subjecting primary neuronal cultures or brain slices, typically from the hippocampus or the cerebral cortex, to oxygen and glucose deprivation (OGD) (Goldberg and Choi, 1993; Martin et al., 1994).

Excitotoxicity is a major hallmark of several neurological disorders, including cerebral ischemia, and is characterized by the accumulation of glutamate in the extracellular space, leading to overactivation of glutamate receptors and the consequent induction of neuronal death. One key factor in excitotoxic cell death is the intracellular Ca^{2+} overload

Abbreviations: AMPAR, α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; AP2, Clathrin adaptor protein 2; CaMKIIα, Ca^{2+}-calmodulin kinase IIα; LDH, Lactate dehydrogenase; MK-801, (5S,10R)-(+)-5-methyl-10,11-dihydro--5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDAR, N-methyl-D-aspartate receptor; nNOS, Neuronal nitric oxide synthase; NO, Nitric oxide; OGD, Oxygen–glucose deprivation; PSD95, Post-synaptic density 95.

* Corresponding author at: Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal.
E-mail address: alc@cnc.uc.pt (A.L. Carvalho).

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(Arundine and Tymianski, 2003; Forder and Tymianski, 2009). Evidence in the literature suggests that both N-methyl-D-aspartate receptors (NMDARs) (Arundine and Tymianski, 2004; Hardingham et al., 2002) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs), specifically Ca^{2+}-permeable-AMPARs (Gorter et al., 1997; Liu et al., 2004; Pellegrini-Giampietro et al., 1997) are responsible for the excitotoxic component of ischemic insults. The NMDARs, in particular, have long been implicated in excitotoxic phenomena, due to their high Ca^{2+} permeability (Goldberg and Choi, 1993; Wang and Qin, 2010). The NMDARs are ionotropic glutamate receptors that form tetrameric structures assembled from two obligatory GluN1 subunits and two GluN2 (A–D) or GluN3 (A, B) subunits (Traynelis et al., 2010). Most NMDAR complexes in the cortex and hippocampus are composed of GluN2A and/or GluN2B in combination with GluN1 subunits. In rodents, the GluN2 subunits are differentially regulated throughout development, with GluN2B being highly expressed from embryonic development and GluN2A expression increasing around the second post-natal week (Monyer et al., 1994; Watanabe et al., 1992). The role of these two subunits in excitotoxicity has remained controversial, as some reports suggest that both subunits contribute to neuronal death (Stanika et al., 2009; Zhou et al., 2013), while others imply a differential contribution of the two subunits (Wyllie et al., 2013). Specifically, GluN2A is thought to contribute to activation of survival signaling cascades (Liu et al., 2007; Terasaki et al., 2010), while GluN2B activates deleterious pathways (Aarts et al., 2002; Martel et al., 2012).

In this work we investigated the impact of the GluN2B subunit on OGD-induced neuronal death. This model correlates more accurately to *in vivo* cerebral ischemia than the excitotoxic insults (usually high NMDA or glutamate concentrations) used in other studies (Choi, 1987; Martel et al., 2012; Stanika et al., 2009). We observed a specific role of this subunit in OGD-induced death, in particular of its C-terminal domain. While GluN2B expression in GluN2B$^{-/-}$ cortical neurons promoted neuronal death in ischemic conditions, GluN2A or the GluN2B subunit with its C-terminal tail swapped with that of GluN2A failed to produce cell death upon OGD. In addition to the neuroprotective effect of disturbing the interaction between GluN2B and post-synaptic density 95 (PSD95), we observed a determinant role of the Ca^{2+}-calmodulin kinase IIα (CaMKIIα)- and clathrin adaptor protein (AP2)-binding sites of GluN2B in mediating OGD-induced neuronal death. We further confirmed the crucial role of the interaction between CaMKIIα and GluN2B subunits in OGD-induced death.

2. Materials & methods

2.1. Materials

The NMDAR antagonist (5S,10R)-(+)-5-methyl-10,11-dihydro-5 H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, #0924) and the GluN2B selective antagonist ifenprodil (#2892) were purchased from Tocris Bioscience (Bristol, UK). The CaMKIIα inhibitor, Autocamtide-2-related inhibitory peptide (AIP, #ALX-151-029-M001) was purchased from Enzo Life Sciences (Farmingdale, NY).

The modified pVIVO2 plasmids expressing nuclear CFP and extracellular YFP-tagged GluN2A, GluN2B, and GluN2B subunits with a mutation in the CaMKIIα-binding site (RQHS → QQHD) were previously described (She et al., 2012). The chimeric GluN2B subunit with the entire tail swapped with that of GluN2A consists of GluN2B (NCBI D10651.2) to Met 829 followed by GluN2A (NCBI NM_012573.3) starting at Ala 829, swapping the subunits within the last transmembrane domain. YFP–GluN2B PDZ mutant encoding the GluN2B subunit with a mutation in the PDZ-binding domain (ESDV → EAAV) was generated using the QuickChange II XL kit (Agilent) and sequence verified. YFP–P2A–myc–GluN2 constructs also express from the light chain human ferritin promoter in modified pVIVO2. These express YFP followed by the self-cleaving P2A peptide [GATNFSLLKQAGDVEENPGP, (Kim et al., 2011)], the signal peptide from GluN2B, a myc tag, and the mature GluN2 sequence. GW-GluN1-1a was also used for COS-7 cell expression (Kim et al., 1996, gift from Doctor Morgan Sheng). The CaMKII WT, CaMKII I205K, which is unable to bind GluN2B subunits and the pSuper shRNA–CaMKIIα constructs (Okamoto et al., 2007) were generous gifts from Doctor Yasunori Hayashi, RIKEN Brain Science Institute, Japan. The rescue construct GFP–CaMKII (Okamoto et al., 2007) was kindly provided by Doctor Paul De Koninck, Université Laval Robert-Giffard, Québec, Canada. The shRNA and the rescue construct have previously been shown to be effective (Okamoto et al., 2007). The control pSuper shRNA (scramble) was a generous gift from Doctor Ramiro Almeida, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal.

2.2. Cell culture and transfection

Two distinct types of neuronal cultures were used in this study. We used cultured cortical neurons from GluN2B$^{-/-}$ (Kutsuwada et al., 1996) and WT littermates (due to limiting numbers of GluN2B$^{-/-}$ hippocampal neurons), or primary cultures of rat hippocampal neurons (Ribeiro et al., 2014) for the localization assays and studies with NMDAR antagonists and CaMKIIα. For the GluN2B$^{-/-}$ and WT littermate cultures, cortical neurons were prepared from embryonic days 17–18 (E17–18) mice, as previously described (Ferreira et al., 2015). Briefly, cortices were dissected and maintained in Hibernate E (Brain Bits, Springfield, IL) supplemented with NeuroCult™ SM1 Neuronal Supplement (StemCell™ Technologies, Grenoble, France) at 4 °C overnight, while genotyping was performed. Tissues from the same genotype were pooled together and dissociated with papain (20 units/ml, 10 min, 37 °C) and deoxyribonuclease I (0.20 mg/ml).

Cortical neurons were electroporated on the day of the culture using the AMAXA system (Lonza, Basel, Switzerland). Briefly, 0.75×10^6 cells were centrifuged and the cell pellet was resuspended in 50 μl of Ingenio™ electroporation solution (MIR50111 — Mirus Bio LLC, Madison, WI) and 2.5 μg of each plasmid was added. After electroporation, neurons were plated on coverslips coated with poly-D-lysine (PDL) in 12 MW plates containing a pre-warmed minimal essential medium (MEM) supplemented with 10% horse serum. Once the neurons attached to the substrate, the plating medium was removed and neurons were placed in Neurobasal® medium (Gibco®, Life Technologies™, Paisley, UK) supplemented with 2% NeuroCult™ SM1, 0.5 mM glutamine, 0.125 μg/ml gentamicin, and insulin (20 μg/ml). Neurons were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air for 15 DIV.

For the localization assays, neuroprotection studies with NMDAR antagonists and CaMKIIα studies, primary cultures of hippocampal neurons were prepared from E18 rats (Fernandes et al., 2014). Briefly, hippocampi were dissected and dissociated with trypsin (1.5 mg/ml Hank's balanced salt solution — HBSS). Neurons were cultured in MW plates with or without glass coverslips, coated with PDL, at a density of 85,000 cells/cm^2 in Neurobasal® medium supplemented with 2% NeuroCult™ SM1 neuronal supplement, 0.5 mM glutamine, 0.125 μg/ml gentamicin and 25 μM glutamate. Neuronal cultures were maintained at 37 °C in a humidified incubator with 5% CO_2/95% air up to 15 DIV.

For expressing YFP–P2A–myc–GluN2 constructs, and for the CaMKIIα overexpression studies, neurons were co-transfected at DIV 9-11 using the calcium phosphate precipitation method (Dahm et al., 2008). Precipitates containing plasmidic DNA were prepared using the following solutions: TE (1 mM Tris–HCl pH 7.3, 1 mM EDTA), 2× HEBS (12 mM dextrose, 50 mM HEPES, 10 mM KCl, 280 mM NaCl and 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.2) and $CaCl_2$ (2.5 M $CaCl_2$ in 10 mM HEPES, pH 7.2). The precipitates were added to the neurons, in the presence of 2 mM kynurenic acid (Sigma-Aldrich, St. Louis, MO), and incubated for 3 h at 37 °C. After the transfection period, the cells were washed with a Neurobasal medium containing 2 mM kynurenic acid

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