Molecular and Cellular Neuroscience 40 (2009) 463–473

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Reverse glial glutamate uptake triggers neuronal cell death through extrasynaptic NMDA receptor activation

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ARTICLE INFO

Article history:
Received 13 June 2008
Revised 6 December 2008
Accepted 20 January 2009
Available online 29 January 2009

Keywords:
Glutamate uptake
Astrocytes
Neurons
Extrasynaptic NMDARs

ABSTRACT

Evidence have accumulated that reverse glutamate uptake plays a key role in the pathophysiology of cerebral ischemia. Here, we investigated the effects of glial glutamate transporter dysfunction on neuronal survival using the substrate inhibitor of glutamate transporters, L-*trans*-pyrrolidine,2-4,dicarboxylate (PDC), that partly mimics reverse glutamate uptake. On mice primary cortical co-cultures of neurons and astrocytes, PDC treatment triggered an elevation of extracellular glutamate concentration, induced neuronal calcium influx and a massive NMDA receptor (NMDAR) mediated-neuronal death without having any direct agonist activity on NMDARs. We investigated the NMDAR subpopulation activated by PDC-induced glutamate release. PDC application led to the activation of both subtypes of NMDARs but the presence of astrocytes was required to activate NMDARs located extra-synaptically. Extrasynaptic NMDAR activation was also confirmed by the loss of neuronal mitochondrial membrane potential and the inhibition of pro-survival p-ERK signalling pathway. These data suggest that reverse glial glutamate uptake may trigger neuronal death through preferential activation of extrasynaptic NMDAR-related pathways.

Introduction

Glutamate, the main excitatory neurotransmitter in the mammalian brain, exerts a dual role in the function of the central nervous system. While normal glutamatergic activity is crucial for the physiological excitatory synaptic transmission, glutamate mediated neuronal death is thought to contribute to all major neurodegenerative disorders. The pivotal point between beneficial or deleterious effects of glutamate may rely on extracellular concentration levels reached (Watkins and Jane, 2006). Therefore extracellular glutamate concentration is tightly regulated by high affinity glutamate transporters named excitatory amino acid transporters (EAATs) (Kanai and Hediger, 1992; Rothstein et al., 1994; for review see Danbolt, 2001). This function is primarily performed by the glial transporter proteins glutamate/aspartate transporter

(GLAST)(Storck et al., 1992; Tanaka, 1993) and glutamate transporter 1 (GLT-1) (Pines et al., 1992). These glial transporters remove glutamate from the synaptic cleft by a sodium/ATP-dependent mechanism (Logan and Snyder, 1972). A neuronal form of these transporters named excitatory amino acid carrier 1 (EAAC1) has been identified (Kanai and Hediger, 1992). However it has been suggested that EAAC1 exerts a preponderant role during the development of central nervous system while its impact on glutamate removal is limited in adults (Nieoullon et al., 2006). Failure of glutamate uptake has been described as a possible mechanism contributing to neuronal death observed in several types of acute injuries. For instance, dysfunction of astrocytic glutamate transporter (Rothstein et al., 1996) has been proposed as a possible mechanism responsible for the well characterized extracellular glutamate accumulation described following cerebral ischemia (Benveniste et al., 1984). Indeed, the deprivation in oxygen and energetic metabolites subsequent to cerebral blood flow interruption leads to a dramatic tissue energy depletion and ionic membranous electrochemical gradient disruption that directly affects astrocyte uptake capacity for glutamate (Madl and Burgesser, 1993). This point was strengthened by the study of Rossi et al. (2000) who showed a significant reduction of ischemic induced-extracellular glutamate

Abbreviations: 4-AP, 4-aminopyridine; Bic, Bicuculline; CREB, cAMP Responsive Element Binding; D-APV, D-2-amino-5-phosphonovalerate; AraC, cytosine beta-D-arabinoside; DIV, Days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; EAAC1, Excitatory amino acid carrier 1; EAATs, Excitatory amino acid transporters; ERK, Extracellular signal Regulated Kinase; E15, embryonic day 15; FBS, Fetal Bovine Serum; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; GLT1, glutamate transporter 1; HBBSS, Hepes and Bicarbonate Buffered Salt Solution; HEK 293, Human embryonic kidney cell line 293; HPLC, High Performance Liquid Chromatography; HS, Horse Serum; LDH, Lactate Deshydrogenase; MAP-2, Microtubule associated Protein-2; MK-801, (+)-5-methyl-10,11-dihydroxy-5h-dibenzo (a,d)cyclohepten-5,10-imine; NMDARs, N-Methyl-D-Aspartate receptors; PDC, L-trans-pyrrolidine-2,4-dicarboxylate; PBS, Phosphate Buffered Saline; DL-TBOA, DL-threo-beta-benzyloxyaspartate.

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doi:10.1016/j.mcn.2009.01.002

efflux by the treatment with selective blockers of glutamate transporters. They suggested that glutamate uptake reversal was partly responsible for the ischemia induced-glutamate efflux. This observation was confirmed by the non vesicular origin of the ischemic induced-extracellular glutamate accumulation (Jabaudon et al., 2000). This elevation of extracellular glutamate concentration results in a sustained activation of glutamatergic receptors leading to neuronal derangements ranging from cell body swelling, dendritic alterations, mitochondrial failure and neuronal death. This "excessive" excitation leading to neuronal death has been named "excitotoxicity" (for review, see Olney, 1990). Excitotoxicity has been described as a mechanism leading to neuronal death observed in acute neuronal injury such as trauma and stroke but also in neurodegenerative diseases (for review see Dirnagl et al., 1999). Excitotoxicity occurs partly because of the overactivation of N-Methyl D-Aspartate receptors (NMDARs) that results in an excessive calcium (Ca^{2+}) influx through the receptor's associated ion channel (Choi, 1988). This unique relationship between NMDAR activation and neuronal death is challenged by their parallel role in neuronal plasticity and neurotrophic processes (Bashir et al., 1991; Bliss and Collingridge, 1993; Nicoll and Malenka, 1999). A key determinant of this duality of NMDAR signalling may rely on the cellular location of the activated receptors. Indeed, while NMDARs are mainly found on the postsynaptic membrane at excitatory synapses (O'Brien et al., 1998; Scannevin and Huganir, 2000) they are not fixed at the synapse. It has been described that some synaptic NMDARs can move laterally to extrasynaptic sites (Rao and Craig, 1997; Tovar and Westbrook, 2002; Choquet and Triller, 2003). Recently, it has been observed that, depending on their subcellular localization, NMDAR activation induces Ca^{2+} pathways that could influence differently the neuronal fate suggesting a functional dichotomy between NMDARs located at the synapse (synaptic receptors) and outside the synapse (extrasynaptic receptors) (Hardingham et al., 2002; Hardingham and Bading, 2003; Zhang et al., 2007). Here, we reproduced the pathological conditions in which glutamate uptake is reversed to evaluate which population of NMDARs may be influenced. Glutamate uptake reversal can be mimicked pharmacologically by EAAT substrate inhibitors, such as L-trans-pyrrolidine-2,4-dicarboxylate (PDC). PDC has been described as a potent substrate inhibitor of glutamate transporters and leads to glutamate uptake reversal by triggering Ca^{2+} independent-glutamate release to the extracellular compartment (Volterra et al., 1996). By promoting glutamate efflux through glutamate transporters reversal, PDC reproduces one of the key early steps leading to neuronal damages during acute brain injury. In the present study, we analyzed the impact of PDC treatment on neuronal fate by determining its impact on synaptic and extrasynaptic NMDAR activation. These experiments were performed on murine primary cortical cultures, a cellular model widely used to study neurons/glia interaction (Volterra et al., 1996; Goldberg and Choi, 1993).

Results

Cellular expression of glutamate transporters

To study the influence of glutamate transporters function on neuronal viability, we used primary murine cortical cultures subjected or not to a treatment with AraC (at 10 μM) to prevent glial proliferation. These types of cultures have been previously characterized as valid cellular models to examine neuronal induced glutamate injury (Rose et al., 1993). However little is known about the expression level of glutamate transporters in such cellular models. By using neuronal (MAP-2) and astrocytic (GFAP) specific immunostaining, we showed that, in the absence of AraC treatment, cortical cultures contain both neurons and astrocytes (Fig. 1A) (47.0% ± 1.75 of astrocytes and 53.0% ± 8.11 neurons) while inhibition of glial proliferation by AraC leads to neuron-enriched cultures containing less than 5% astrocytes (Fig. 1B). Next, we determined the cellular expression of glutamate transporters (GLT-1, GLAST and EAAC1) in these murine cortical cultures of neurons and astrocytes (at 14 DIV). While GLT-1 and GLAST immunolabeling co-localized only with GFAP staining (Figs. 1C and D), EAAC1 displayed an exclusive neuronal expression (Fig. 1E). These results validate the use of these primary cortical cultures to study the impact of glutamate uptake reversal on neuronal fate.

Reverse glutamate uptake increases extracellular glutamate level

PDC is a potent competitive inhibitor of the glutamate transporters, but it is also the only substrate inhibitor known for these transporters. When applied at high concentration, PDC stimulates glutamate efflux from primary cultures of astrocytes (Waagepetersen et al., 2001). Here, we measured by HPLC, the glutamate concentration in the bathing medium of primary cortical cultures containing neurons and astrocytes exposed to 250 μM PDC for 5, 10, 15, 30, 45 min and 1 h. In control condition, glutamate concentration was measured at 1.24 μM ± 0.12 which is similar to the concentration measured by Volterra et al. (1996) in similar culture systems. Application of PDC (at 250 μM) produced a continuous build-up of glutamate concentration that leads to a 3- to 8-fold increase in glutamate concentration, respectively 3.19 μM ± 0.21 at 5 min, 4.03 μM ± 0.68 at 10 min, 5.33 μM ± 0.68 at 15 min, 6.72 μM ± 0.60 at 30 min, 6.96 μM ± 0.43 at 45 min and 8.69 μM ± 0.55 after 1 h (Fig. 2A). These data confirmed that PDC treatment increases extracellular glutamate concentration.

PDC application is highly neurotoxic in co-cultures of neurons and astrocytes

Next, we determined if the corresponding glutamate concentration reached after PDC application for 1 h (approximately 10 μM glutamate) exerts any neurotoxicity. We observed that glutamate exhibits a moderate neurotoxicity in neuron and astrocytes co-cultures (Figs. 2B, C) that is increased in the presence of the glutamate transporter blocker, TBOA (at 30 μM). As expected 10 μM glutamate was equally neurotoxic in the presence or absence of DL-TBOA on near pure neuronal cultures. Together these data indicate the major role of glial glutamate uptake systems in preventing glutamate toxicity (Fig. 2C). Moreover, PDC-mediated cell death was not modified by tetrodotoxin (TTX) in neither non-AraC nor AraC treated cultures confirming the absence of contribution of presynaptically released glutamate in the PDC-mediated toxicity (Fig. 2B). It is well known that NMDAR overactivation leads to excitotoxic necrotic death (Choi, 1988). Then, we studied the involvement of NMDARs in the PDC-driven neurotoxicity. Co-cultures of neurons and astrocytes were treated for 1 h with either 15 μM NMDA or 250 μM PDC. NMDA application produced a significant neuronal death (73.7% ± 3.9) and PDC induced a large neurotoxicity (91% ± 3.9) that was fully blocked by co-application of the competitive NMDA antagonist D-APV (13.3% ± 1.4) (Fig. 2D). Immunolabeling with MAP-2 and GFAP confirmed that, while PDC triggered massive neuronal death, it did not affect astrocyte viability (Fig. 2E). Taken together these data reveal that PDC-induced neurotoxicity is a NMDAR-dependent mechanism. Next, we determined if, in primary cortical cultures treated or not with AraC, PDC treatment (for 5 min) at increasing concentrations is neurotoxic. We applied PDC at increasing concentrations (50, 100, 150, 200 and 250 μM). Neuronal cell death was assessed 24 h later. Co-cultures of neurons and astrocytes displayed a significant neuronal death whereas near pure neuronal cultures were significantly less vulnerable to PDC applications (Fig. 2F). This reveals that PDC-induced neurotoxicity involves reverse astrocytic glutamate uptake.