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Dysregulated glutamate uptake by astrocytes causes oligodendroglia death in hypoxic perventricular white matter damage ☆

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ABSTRACT

Excess glutamate mediates damage to oligodendroglia, resulting in myelination disturbances characteristic of hypoxic periventricular white matter (PWM) damage. We sought to examine if hypoxia altered the expression of astroglial excitatory amino acid transporters (EAAT1, EAAT2 and EAAT3) in the PWM, and, if so, whether it activated astroglial N-methyl D-aspartate receptors (NMDAR) which might lead to apoptosis of oligodendroglia. EAAT expression in the PWM of neonatal rats was measured at different time points after hypoxic exposure; it was attenuated at 7 and 14 d following hypoxia. Hypoxia prevented the uptake of glutamate by astroglial EAATs causing increased levels of extracellular glutamate. Excess glutamate augmented the expression of functional astroglial NMDAR. Following hypoxia, an increase in gap junction proteins between astroglia and oligodendroglia aided in the spreading of NMDAR-mediated excitotoxic calcium signals into the latter cell type triggering its apoptosis. Hence, dysregulated glutamate homeostasis is believed to contribute to hypoxia-induced death of oligodendroglia leading to neonatal PWM damage.

Introduction

Periventricular white matter (PWM) damage (PWMD) as a consequence of hypoxia, is one of the major causes of neurological deficits in premature newborn, with some cases resulting in cerebral palsy (Hack and Merkatz, 1995; Peterson et al., 2000). Although several mediators of PWMD have been identified, excess glutamate is considered to be a cardinal mediator of oligodendroglia death under hypoxic conditions (Follett et al., 2004; Volpe, 2001). Glutamate balance in the brain is achieved by a family of sodium-dependent astroglial glutamate transporters, which bind to and subsequently uptake glutamate (Robinson and Dowd, 1997). The key glutamate transporters include excitatory amino acid transporter 1 (EAAT1) [GLAST glutamate-aspartate transporter], EAAT2 [GLT-1 glutamate transporter 1] and EAAT3. Astroglial regulation of glutamate homeostasis is affected under several pathological conditions. Downregulated expression of EAATs has been reported following hypoxic–ischemic insults and traumatic brain injury resulting in the accumulation of extracellular glutamate (Boycott et al., 2007; Rao et al., 1990). Glutamate uptake being an energy-dependent process, energy compromising events such as hypoxia, reverse the function of glutamate transporters, further augmenting astroglial glutamate release (Allen et al., 2004; Danbolt, 2001; Malarkey and Parpura, 2008; O'Shea, 2002). The present study aimed at examining the expression and function of astroglial glutamate transporters EAAT1, EAAT2 and EAAT3 in hypoxic PWM and its implication in excitotoxicity to oligodendroglia.

Excitotoxicity is the accumulation of excitatory neurotransmitters like glutamate and the overstimulation of their corresponding receptors resulting in increased Ca^{2+} influx into the cell (Olney, 1989). Glutamate mediated excitotoxicity occurs *via* activation of glutamate receptors, mainly, N-methyl D-aspartate receptor (NMDAR). It has been shown that glutamate receptor activation may regulate the expression of astroglial glutamate transporters (López-Bayghen et al., 2003; Rodrigo and Felipo, 2007). However, in pathological conditions, it is proposed that glutamate transporter dysfunction precedes glutamate receptor activation (Napier et al., 2012). Hence, we hypothesized that hypoxia-induced increase in glutamate due to dysregulated EAAT function might augment the expression of NMDAR in astroglia. To ascertain this, we investigated whether hypoxia/glutamate treatment induced the cell-surface expression of astroglial NMDAR *in vitro*. This was followed by studying the effect of glutamate transporter on NMDAR activation and associated influx of calcium.

Against the above background, it is relevant to note the presence of functional gap junctions between astroglia and oligodendroglia in the developing PWM (Maglione et al., 2010) and that these junctions are critical for normal glial function (Magnotti et al., 2011). Indeed, calcium signaling occurs *via* gap junctions formed between connexin 43 (Cx43) in astroglia and connexin 47 (Cx47) in oligodendroglia (Parys et al., 2010) that remain open following hypoxia (Cotrina et al., 1998). In light of the above, we surmised that activation of

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astroglial NMDAR in hypoxic conditions might cause excitotoxic damage to oligodendroglia *via* gap junctions. To this end, we examined if astroglia increased intercellular crosstalk with oligodendroglia *via* gap junctions under hypoxic conditions by using a gap junction inhibitor, carbenoxolone disodium (CBN). Additionally, we have used DL-threo ß-benzyloxyaspartic acid (TBOA) – a competitive EAAT inhibitor, and MK801 – an NMDAR antagonist to substantiate the involvement of astroglial EAATs and NMDAR in hypoxic PWMD.

Results

Cellular localization and expression of EAAT1, EAAT2 and EAAT3 in control and hypoxic PWM

Significant differences in protein expression of EAAT1, EAAT2 and EAAT3 in the PWM were observed between control and hypoxic groups (Fig. 1). In the PWM of 7 d old rat brain (Fig. 1A–C), EAAT immunoexpression was localized in the astroglia as identified by double labeling with GFAP (Fig. 1A, B, C [c, f]). EAAT1 (Fig. 1A [b, e]), EAAT2 (Fig. 1B [b, e]) and EAAT3 (Fig. 1C [b, e]) immunofluorescence appeared attenuated at 7 d following hypoxia in comparison with the matching control groups. The expression of EAATs was found to be developmentally upregulated. The immunoreactive bands of EAAT1, EAAT2 and EAAT3 were detected at 60 kDa, 62 kDa and 57 kDa respectively (Fig. 1G). EAAT1 (Fig. 1D) and EAAT2 (Fig. 1E) protein levels were significantly decreased at 7 and 14 d after hypoxia when compared with the controls. The protein expression of EAAT3 was down regulated at 3 h, 7 and 14 d following hypoxic exposure but it was comparable to the controls at other time points (Fig. 1F).

Hypoxia decreased protein expression of glutamate transporters in astroglial cultures

Significant differences in protein expression of EAATs were observed in primary astroglial cultures between the control and hypoxic groups (Fig. 2). Double immunofluorescence studies showed colocalized expression of EAATs in astroglial cells, marked by GFAP. At 24 h following hypoxia (2 h), EAAT1, EAAT2 and EAAT3 immunoexpressions in GFAP-positive astroglia were noticeably reduced in comparison to the control cells (Fig. 2A, B, C [b, e]). The cultures were exposed to 2 h of hypoxia and the protein levels were evaluated at different time points following re-oxygenation. EAAT1 and EAAT2 protein levels were significantly decreased at 1, 3, 12 and 24 h post-hypoxic conditioning (Fig. 2D and E). EAAT3 protein expression was down regulated at all time points notably at 1, 12 and 24 h after hypoxic conditioning (Fig. 2F). Also, a notable reduction in expression of EAAT1, EAAT2 and EAAT3 was observed in control groups. This might be due to serum deprivation in culture medium (Fig. 2D–F).

Hypoxia augments glutamate release

The extracellular glutamate in the supernatant of primary astroglial cultures exposed to hypoxia was found to be significantly increased (Fig. 3A). The addition of TBOA, an EAAT inhibitor, caused a significant decrease in extracellular glutamate in hypoxia group. This suggests that hypoxia-induced dysregulated expression and function of astroglial EAATs potentiated the release of glutamate. However, the addition of TBOA to the control cells led to increased extracellular glutamate.

Hypoxia-induced glutamate release potentiates expression of NMDA receptors in astroglia

Since hypoxia-induced dysregulation of the glutamate transporter system resulted in excess extracellular glutamate, we extended the study to determine the effect of hypoxia/glutamate on the expression of NMDAR in astroglia. NMDAR1 subunit is present in all NMDARs and hence was used as an indicator for NMDAR expression. Exposure of primary astroglial cells to hypoxia resulted in upregulated mRNA expression of NMDAR1 subunit (Fig. 3B). Incubation of primary astroglia with glutamate also increased NMDAR1 mRNA expression. Western blot analysis showed a significant increase in the cell surface expression of NMDAR1 following hypoxia/glutamate exposure (Fig. 3C). The expression of cell surface NMDAR1 (Fig. 3C, E, F) and total NMDAR1 (Fig. 3D, E, F) correlated with glutamate levels (Fig. 3A) as measured in the culture medium derived from primary astroglia of the respective treatment group. *In vivo* studies showed that hypoxia-induced excess glutamate caused an increase in NMDAR1 expression in astroglia in the PWM, which was prevented by TBOA (Fig. 4).

Hypoxia potentiates expression of gap junctions in astro-oligodendroglia both in vitro and in vivo

Hypoxia induced an increase in expression of gap junction proteins, connexins, Cx43 and Cx47 in astroglia and oligodendroglia, respectively. Western blotting and double immunofluorescence studies done *in vivo* (Fig. 5) and *in vitro* (Fig. 6) indicated a significant increase in Cx43 expression localized in astroglia and that of Cx47 in oligodendroglia in hypoxic groups as compared with their corresponding controls. This suggests an increase in number of gap junctions (Cx43–Cx47) between astroglia and oligodendroglia following hypoxic conditioning.

Hypoxia-induced increase in calcium influx in astroglia and oligodendroglia

Live intracellular calcium in primary co-cultures of astroglia and oligodendroglia was imaged using Rhod 2AM dye. A notable increase in cytosolic calcium was observed in both astroglia (CC1 negative cells) and oligodendroglia (CC1 positive cells) following exposure of co-cultures to hypoxia (Fig. 7A (e), B). Administration of MK801 and TBOA in hypoxic groups significantly prevented the calcium influx in both cell types (Fig. 7A (f, g)). Following the addition of CBN (a gap junction inhibitor) to the co-cultures exposed to hypoxia, there was a significant decrease in oligodendroglia cytosolic calcium; however, no significant reduction was observed in astroglia (Fig. 7A (h)). Also an increase in intracellular calcium in astrocytes in control groups administered with TBOA was noted (Fig. 7A (b), B); this is consistent with the increased NMDAR expression observed in C + TBOA treatment group (Fig. 3B).

Astroglial NMDAR activation in excitotoxic death to oligodendroglia via gap junctions

Measurement of cysteine aspartic acid-specific protease (caspase) 3/7 activities in primary co-cultures of astroglia and oligodendroglia indicated an increase in caspase 3/7 activity following hypoxia, which was prevented by the addition of TBOA, MK801 and CBN (Fig. 8A). Double immunofluorescence studies showed caspase 3 to be specifically expressed in oligodendroglia in co-cultures of astroglia and oligodendroglia (Fig. 8B). Caspase 3 immunoexpression was increased in oligodendroglia exposed to hypoxia (Fig. 8B [e]) and decreased significantly in hypoxic groups treated with TBOA, MK801 and CBN (Fig. 8B [h, k, n]). Control groups treated with drug showed no significant difference in comparison to control (immunofluorescence data not shown).

Discussion

Glutamate is a major excitatory neurotransmitter found abundantly in the brain (Fonnum, 1984). Elevated levels of glutamate have been implicated in various neurological disorders, including