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Research Report

Ontogeny of AMPA and NMDA receptor gene expression in the developing sheep white matter and cerebral cortex

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Abstract

This study examined the hypothesis that the high prevalence of white matter injury in premature infants is associated with increased expression of calcium-permeable forms of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors in pre-myelinating white matter. We characterized expression of subunits of the AMPA, and for reference, the *N*-methyl-D-aspartate (NMDA), glutamate receptors at 0.5, 0.65, 0.85, and term gestation in the ovine fetal white matter and cerebral cortex. There was a low expression of the critical calcium-impermeable AMPA receptor GluR2 subunit in subcortical white matter both absolutely and relative to other AMPA subunits throughout gestation. In contrast, GluR2 subunit mRNA expression fell in the cerebral cortex with increasing gestation whereas protein expression increased. These findings suggest a vulnerability of subcortical white matter to AMPA receptor-mediated calcium toxicity throughout the second half of gestation. Thus, the hypothesis that AMPA receptor-mediated glutamate toxicity contributes to brain damage in premature infants needs to be revised.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Excitatory amino acid receptors: structure, function, and expression

Keywords: NMDA receptor; AMPA receptor; White matter; Cerebral cortex; Excitotoxicity

1. Introduction

Excessive intracellular Ca^{2+} influx following activation of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptor is toxic to both oligodendrocytes and neurons in vitro [25,31], and is thought to be an important mechanism of brain cell injury following asphyxia. The permeability of AMPA receptors to Ca^{2+} is regulated by the presence or absence of the critical Ca^{2+}-impermeable AMPA receptor GluR2 subunit. Thus, the lower the expression of GluR2 compared to other AMPA receptor subunits, the greater the likelihood of formation of AMPA receptors which are Ca^{2+} permeable and the greater the vulnerability to AMPA receptor-mediated cell death [40]. Infants born before 32 weeks of gestation have a very high incidence of periventricular white matter damage (PVL) [48]. This early white matter damage is followed by post-injury gray matter transformations, with reduced gray matter volumes at term, which are believed to mediate the neurological complications of prematurity [21,27]. The age of maximum risk of white matter injury precedes the onset of axonal myelination [4], and has been related to intrinsic vulnerability of pre-myelinating oligodendrocytes to oxida-

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tive stress and local accumulation of excitatory amino acids (excitotoxins) [3,5,12]. Recent in vitro studies raise the possibility that a transient period of over-expression of Ca^{2+}-permeable AMPA receptors may mediate this apparent vulnerability of pre-myelinating oligodendrocytes to ischemia compared with mature cells [22].

In the present study, we examined the hypothesis that the pre-myelinating subcortical white matter of the preterm brain would exhibit an increase in expression of Ca^{2+}-permeable AMPA receptors. We characterized AMPA receptor subunit (GluR1, 2, 3, and 4) mRNA expression and GluR2 protein levels in the subcortical white matter and parasagittal cortex of the fetal sheep brain from 0.5 gestation to term. For comparative purposes, we also examined mRNA expression of the *N*-methyl-D-aspartate (NMDA) receptor 1 subunit (NR1), which is essential for the formation of a functional NMDA-type glutamate receptor [36]. The NMDA receptor is thought to be an important mediator of neuronal cell death in the immature brain [35].

2. Materials and methods

2.1. Animals

Time-mated pregnant Romney sheep were killed by an overdose of sodium pentobarbital as approved by the Animal Ethics Committee of the University of Auckland. Fetal brains were collected at day (d) 70 (0.5 gestation), d94 (0.65 gestation), d125 (0.85 gestation), and d140 (term gestation). The brain maturation of the 0.5, 0.65, and 0.85 gestation fetal sheep are broadly equivalent to the 23- to 24-week, 28-week, and term human infant, respectively. Cortical myelination starts after 0.7 gestation, equivalent to the 34-week human brain [39]. Peak brain growth in the sheep occurs at 0.85 gestation, and at term in man [11]. At full term, the precocial sheep brain is highly myelinated, comparable to the 1- to 2-year-old human infant [33,39].

2.2. Relative quantitative RT-PCR analysis

Unfixed brains from fetal sheep at d70 ($n = 4$), d94 ($n = 7$), d125 ($n = 4$), and d140 ($n = 6$) were slow-frozen on dry ice and stored at −80 °C. Brain tissues were then thawed to −18 °C and cryostat-sectioned (80 μm), beginning at the level of the optic chiasm according to the stereotaxic atlas for the fetal sheep [16]. Coronal tissue sections were thaw-mounted onto poly-L-lysine-coated microscope slides. The slides were desiccated and stored at −80 °C until tissue micropunch. Tissue micropunches were taken from the subcortical white matter and parasagittal region of the cerebral cortex.

Micropunch needles were prepared from 18-gauge hypodermic needles (internal diameter 0.90 mm) [9]. Each needle was used for only one series of punches per area per animal. For each animal, four punches from each target area were taken per section. Punches from five sequential sections were pooled to give a total of 20 punches per area. The tissue punches were expelled from the needle with 400 μl ice-cold TRIzol reagent (Life Technologies Gibco BRL, Rockville, USA) into a 1.5-ml eppendorf tube (Axygen Scientific, Union City, USA) and sonicated for 10 min in an ice-cold water bath [18].

Total RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions. The RNA pellet was re-dissolved in 20 μl diethyl pyrocarbonate (DEPC)-treated water and stored at −80 °C. RNA integrity was determined by agarose gel electrophoresis. 10 μl of RNA was reverse transcribed in a 20-μl final reaction mixture with 200 U SuperScript II reverse transcriptase and 50 ng random hexamers (Invitrogen Life Technologies, Carlsbad, USA). Oligonucleotide primers for PCR amplification of individual target cDNA were specific to the regions which showed homology between the rat and human genes [15]. Primers were also designed to amplify regions spanning intron/exon boundaries to control for genomic contamination, and the region selected was common to all splice variants. Forward and reverse primers (Invitrogen Life Technologies) used are listed in Table 1, together with primer annealing temperatures and the length of amplified products. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AY346122 (GluR1), AY346123 (GluR2), AY346124 (GluR3), AY346125 (GluR4), and AY346126 (NR1).

18S rRNA was used as an internal control with a QuantumRNA primer-Competimer set (Quantum*RNA*™ Universal 18S Internal Standard Kit, Ambion, Austin, USA) to allow relative quantitative analysis of target genes. Pilot experiments determined the correct ratio of primers to competimers and cycle number to yield multiplex PCR products within the linear range of amplification. The PCR

Table 1
Oligonucleotide primers and PCR conditions

Subunit	Upstream primer (5′-3′)	Downstream primer (5′-3′)	T_a (°C)	Size (bp)
GluR1	TGGTGGTTCTTCACCCTGATCAT	TATGGCTTCATTGATGGATTGC	60	707
GluR2	TGGTGGTTCTTCACCCTGATCAT	TGCAAAATTCTGGGAATTCTGC	60	726
GluR3	TGGTGGTTCTTCACCCTGATCAT	AATTCTGAGTGTTGGTGGCAGG	60	724
GluR4	CTGGCCTATGAGATTTGGATG	ACCCTTGGGCGTTGCTAC	55	657
NR1	GGAGCGGGTGAACAACAGCAACAA	AGCACCGCCGAGTCCCAGATGAAG	63.3	747

Oligonucleotide sequences used as primers in PCR reactions. Reaction parameters were empirically determined. T_a = annealing temperature. Product sizes are indicated in base pairs (bp).