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Research article

Ionotropic glutamate receptor expression in human white matter

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HIGHLIGHTS

- iGluRs are expressed in normal human white matter.
- AMPA receptor subunit GluA4 is expressed on oligodendrocytes, myelin and on axons.
- NMDA receptor subunit GluN1 is expressed on oligodendrocytes and myelin.
- Results confirm findings reported in the literature from rodent white matter.

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ABSTRACT

Glutamate is the key excitatory neurotransmitter of the central nervous system (CNS). Its role in human grey matter transmission is well understood, but this is less clear in white matter (WM). Ionotropic glutamate receptors (iGluR) are found on both neuronal cell bodies and glia as well as on myelinated axons in rodents, and rodent WM tissue is capable of glutamate release. Thus, rodent WM expresses many of the components of the traditional grey matter neuron-to-neuron synapse, but to date this has not been shown for human WM. We demonstrate the presence of iGluRs in human WM by immunofluorescence employing high-resolution spectral confocal imaging. We found that the obligatory N-methyl-D-aspartic acid (NMDA) receptor subunit GluN1 and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA4 co-localized with myelin, oligodendroglial cell bodies and processes. Additionally, GluA4 colocalized with axons, often in distinct clusters. These findings may explain why human WM is vulnerable to excitotoxic events following acute insults such as stroke and traumatic brain injury and in more chronic inflammatory conditions such as multiple sclerosis (MS). Further exploration of human WM glutamate signalling could pave the way for developing future therapies modulating the glutamate-mediated damage in these and other CNS disorders.

1. Introduction

The human CNS expresses a complex array of neurotransmitters and receptors to support electrochemical communication between neurons. The major excitatory transmitter in the mammalian CNS is glutamate, which binds to a variety of ionotropic and metabotropic receptors at synaptic and extra synaptic sites on postsynaptic neurons [10,35,41,65]. Glutamatergic signalling supports critical CNS functions such as learning, memory, motor control and sensory processing. However, excessive levels of glutamate are implicated in a wide variety of human CNS disorders including stroke, trauma, epilepsy, and degenerative conditions such as Alzheimer's disease and even in neuroinflammatory disorders such as multiple sclerosis (MS) [15,25,34,35,38,64].

Neuron-to-astrocytic communication via AMPA and NMDA receptors has also been observed [28] indicating that glutamate-dependent signalling is not restricted to inter-neuronal neurotransmission. Nor is glutamate receptor expression restricted to grey matter neurons; CNS glia, both of the astrocytic and oligodendrocytic lineages, express the same ionotropic glutamate receptors (iGluRs), as do neurons [23,33,36,63]. The role of glial receptors is less clear but axo-glial signalling is suggested by observations that axons in the corpus callosum release glutamate in an activity-dependent manner to signal oligodendrocyte precursor cells (OPCs)

Abbreviations: (AMPA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; (CNS), central nervous system; (DAPI), 4′,6-diamidino-2-phenylindole; (GABA), gamma-aminobutyric acid; (iGluR), ionotropic glutamate receptor; (MBP), myelin basic protein; (MS), multiple sclerosis; (NMDA), N-Methyl-D-Aspartic Acid; (OPC), oligodendrocyte precursor cell; (WM), white matter.

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via AMPA receptors [26,70]. In addition, NMDA receptor-dependent signalling appears to play a role in axonal myelination by oligodendrocytes [32]. These receptors, expressed on processes of oligodendrocytes and in the mature myelin sheath itself [36,46] may also play a pathophysiological role during ischemia or trauma where demyelination is prominent [2,23,36,59]. Most recently, we have shown activity-dependent signalling between electrically active central axons and their overlying myelin sheaths supported by vesicular glutamate release from internodal axons, which activates myelinic NMDA and AMPA receptors [37]. Central myelinated axons also express iGluRs. Using an *ex vivo* rodent spinal cord model, Ouardouz et al. demonstrated the presence of AMPA and kainate receptors on dorsal column axons, that are functionally coupled to axonal Ca^{2+} stores [39,40] (for review see [56]). The origin of glutamate release in the WM likely includes axons and possibly astrocytes [24,37,42].

From animal experiments it has become clear that neuronal and glial WM elements express a variety of glutamate receptors. What is not known is whether human WM has a similar complement of these receptors, which was the goal of this report. This has important implications for our understanding of mechanisms of human WM disorders where axonal degeneration and demyelination may be very prominent.

2. Materials and methods

2.1. Immunohistochemistry

The Clinical Research Ethics Board at The University of British Columbia approved this work. Five μm thick formalin-fixed paraffin sections of human frontal cortex and underlying WM from 9 human brains (see Table 1) were placed on charged microscope slides.

The sections were deparaffinized by immersion in 100% xylene (Surgipath, Richmond, IL), for 5 min 3 times followed by 5 min 3 times in 100% ethanol (Surgipath, Richmond, IL), 5 min in 95% ethanol and rinsed in distilled water. Antigen retrieval was performed by microwaving slides for 10 min in 10 mM sodium citrate (Sigma-Aldrich, Oakville, ON, Canada) in distilled water (pH 6) for 2 min at 90% power, 2 min at 70% power, and 6 min at 50% power. Slides were allowed to cool for 20 min before washing for 5 min in phosphate buffered saline (PBS, pH 7.4). Slides were then immersed in 1% sodium borohydride (pH 7.5; Sigma-Aldrich, Oakville, Canada) in PBS for 20 min and washed 5 min 3 times in PBS before Fc-receptor blocking in 5% normal donkey serum (Sigma-Aldrich, Oakville, ON, Canada) in PBS for 30 min. Slides were incubated overnight at room temperature with the following primary antibodies in a cocktail: rabbit anti-NMDA receptor subunit GluN1 (1:100, Abcam, Toronto, ON, Canada) or goat anti-AMPA receptor subunit GluA4 (1:50, Abcam, Toronto, ON, Canada) together with mouse anti-neurofilament (1:250, Covance, Montreal, QC, Canada) and goat anti-myelin basic protein (MBP; 1:400, Santa Cruz Biotechnology, Dallas, TX) or rabbit anti-human MBP (1:1000, Dako, Glostrup, Denmark). For control slides, immunoglobulin fractions in the same concentrations as the primary antibodies from rabbit (Dako, Glostrup, Denmark); mouse (1:250, Dako, Glostrup, Denmark) and goat (Sigma-Aldrich, Oakville, ON, Canada) were substituted for the appropriate primary antibodies. The following day, sections were washed 5 min 3 times in PBS with 0.03% Tween 20 (Fisher Scientific, Fair Lawn, NJ) before incubation with a mixture of secondary antibodies (Alexa Fluor 488 donkey anti-rabbit or Alexa Fluor 488 donkey anti-goat together with Alexa Fluor 647 donkey anti-mouse and Alexa Flour 568 donkey anti-goat or Alexa Fluor 568 donkey anti-rabbit (all 1:300, Life Technologies, Burlington, ON, Canada) for 3–4 h. Slides were washed 5 min 5 times in PBS with 0.03% Tween 20 for 5 min 3 times before 5 min incubation with 30 nM 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain in PBS (Life Technologies, Burlington, ON, Canada). DAPI stain was followed by 3 times 5 min wash in PBS with 0.03% Tween 20 and mounted with Prolong Gold mounting media (Life Technologies, Burlington, ON, Canada). Slides were allowed to air-dry over night and then stored in a cool, dark place until spectral confocal microscopy was performed.

For the simultaneous demonstration of NMDA receptor subunit GluN1 and oligodendrocytes, the rabbit anti-NMDA receptor subunit GluN1 antibody (1:100, Abcam, Toronto, ON, Canada) was used in a similar immunofluorescence procedure, combining it with a goat antibody to Sox10 (1:100, R&D Systems, Minneapolis, MN), a marker that recognizes cells of the oligodendroglial lineage from the early precursor stages to maturity [71]. Secondary antibodies consisted of Alexa Fluor donkey anti-rabbit 488 and Alexa Fluor donkey anti-goat 568 (both at 1:300, Life Technologies, Burlington, ON, Canada). DAPI was employed as a nuclear counterstain. Autofluorescence was blocked with Sudan Black (Fisher Scientific, Ottawa, ON, Canada), 0.3% in 70% ethanol. Co-localization of NMDA receptor subunit GluN1 and Sox10 was observed on a Leica DM4000 B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and representative images, with contrast enhancement, were taken on a Zeiss Axio-observer Z1 spinning-disc confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany).

2.1.1. Spectral confocal microscopy

Given the significant spectral overlap of the fluorophores employed, and the fact that quadruple staining was done, crosstalk between color channels was a concern. Therefore, to reduce this effect, which would result in potentially incorrect interpretation and co-localization, slides were imaged in spectral mode on an inverted Nikon D-Eclipse C1si confocal laser-scanning microscope with a 60×1.4 NA oil immersion objective. The spectral detector captures the entire emission spectrum of all fluorophores in 32 spectral channels (10 nm width) over a range of 415–725 nm. Spectral images were quantitatively unmixed and separated into individual channels using ImageTrak (written by PKS; http://www.ucalgary.ca/styslab/imagetrak) and are shown as RGB color overlays. The sources for excitation were 403, 561 and 637 nm diode lasers, and a 488 nm argon laser.

3. Results

Although representative figures are shown, the findings were similar in all 9 cases, as detailed below.

3.1. Oligodendrocyte cell bodies, processes and myelin express the NMDA receptor subunit GluN1

GluN1 immunoreactivity was widely expressed in the WM in samples from all patients. We found GluN1-positive staining in the cytoplasm of WM glia. These glial cells uniformly had small round nuclei, expressed small amounts of MBP, and were strongly positive for Sox10, features that are characteristic of oligodendrocytes (Fig. 1C, Suppl Fig. S1). In addition, GluN1 staining was evident as small foci within myelin sheaths (Fig. 1C). More commonly, GluN1 was found in glial processes abutting the myelin sheath. In these instances, the point of contact with the myelin demonstrated co-localization with MBP (Fig. 1A, B, D), consistent with the juxtaposition and continuity of a GluN1-positive oligodendrocyte with the adjacent myelin sheath. GluN1 positivity was not evident in any axons. Control staining (Fig. 1E–G; IgGs replacing primary anti-