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EXPRESSION OF THE EXON 9–SKIPPING FORM OF EAAT2 IN ASTROCYTES OF RATS

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Abstract—mRNA for the exon 9–skipping form of the glutamate transporter excitatory amino acid transporter (EAAT) 2 (glutamate transporter 1, GLT-1) is known to be expressed in brain and spinal cord, and such expression was initially proposed to be associated with motor neuron disease. Surprisingly, a protein corresponding to the size of this splice variant has not previously been detected when using antibodies against one of the possible carboxyl terminal regions of EAAT2. This has been construed as indicating that little of the exon 9–skipping protein is expressed, or that such protein is not stable. We have now made selective antibodies against the splice site of this form of EAAT2. We show that in the adult rat brain and spinal cord, it is expressed primarily in populations of white matter astrocytes. Astrocytes expressing this splice variant also expressed glial fibrillary acidic protein. Expression was developmentally regulated, being expressed in a small number of astrocytes at postnatal day 7, but strongly expressed by large populations of white matter astrocytes by 25 days postnatum and into adulthood. Only a subset of gray matter astrocytes and radial glia expressed exon 9–skipping EAAT2. We suggest that exon 9–skipping EAAT2 may have a role in regulating extracellular glutamate in white matter tracts, either by interacting with normally spliced EAAT2 and modifying its targeting or transport activity, or by acting as a transporter itself. Conversely, the limited expression in gray matter suggests it is unlikely to be important for modulating synaptic levels of glutamate.

Key words: glutamate transporter, EAAT, ALS, white matter, astrocyte, excitotoxicity.

Glutamate homeostasis in the brain is achieved via the actions of multiple glutamate transporters. Excitatory amino acid transporter (EAAT) 2, also known as glutamate transporter 1 (GLT-1), is one of the two most abundant glutamate transporters in the adult CNS (Chaudhry et al., 1995; Lehre et al., 1995; Williams et al., 2005). The exon 9–skipping form of EAAT2 was cloned from human tissues (Lin et al., 1998; Meyer et al., 1998, 1999) and was initially described (Lin et al., 1998) as being selectively present in brains from patients that had been afflicted by motor neuron disease (amyotrophic lateral sclerosis) leading to the proposal that expression of this splice variant was a causal factor in the pathogenesis of this disease. Studies by other groups have not made this association since it is also prevalent in control populations (Meyer et al., 1999; Honig et al., 2000; Ludolph et al., 2000; Flowers et al., 2001). Surprisingly, there has been no evidence to date that mRNA for the exon 9–skipping form of EAAT2 is translated to form protein. Initial reports (Lin et al., 1998) indicated that Western blotting with an antibody against the carboxyl terminal region of the originally described form of EAAT2 (which we refer to as GLT-1α), did not reveal the expected lower molecular weight band that would correspond to the exon 9–skipping form of EAAT2. Accordingly it was posited that the protein might be expressed albeit at low levels and with limited stability, but that such protein might interact with normally spliced protein, thereby influencing its abundance by destabilizing oligomeric complexes or other mechanisms (Lin et al.,1998).

While GLT-1α is an abundantly expressed carboxyl terminal splice variant of GLT-1 in the rat brain, in the human brain only a small part of the total GLT-1 pool is demonstrable with antibodies to this epitope (Williams et al., 2005). This raised the possibility that the exon 9–skipping form of EAAT2 might have an alternate C-terminal region and thus not be detectable with antibodies against GLT-1α. Accordingly, we hypothesized in this study that protein encoded by the exon 9–skipping form of EAAT2 might be expressed in the brain. The expression of protein encoded by exon-skipping variant forms of EAAT1 has recently been investigated in our laboratory, including the exon 9–skipping form of EAAT1 (Macnab et al., 2006; Macnab and Pow, 2007; Sullivan et al., 2007). We have demonstrated that it is possible to generate antibodies selective for the new splice sites, and to detect EAAT1 in novel locations. Accordingly in this study we have used the same approach and generated antibodies against the predicted splice site of the exon 9–skipping form of EAAT2. Our aim was to determine firstly whether protein encoded by the splicing of EAAT2 is expressed, and secondly to determine if labeling for such was coincident with that for GLT-1α.

EXPERIMENTAL PROCEDURES

Animal experiments complied with the guidelines of the NHMRC (Australia) and in accord with the NIH Guide to the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Antibodies were generated by immunizing rabbits according to our standard protocols (Pow et al., 2003), using the peptide H_2N-QIVTVRDRMRT (referred to hereafter as peptide 1), which spans the splice region between exons 8 and 10 of EAAT2

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Abbreviations: BSA, bovine serum albumin; EAAT, excitatory amino acid transporter; GFAP, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; P, postnatal day.

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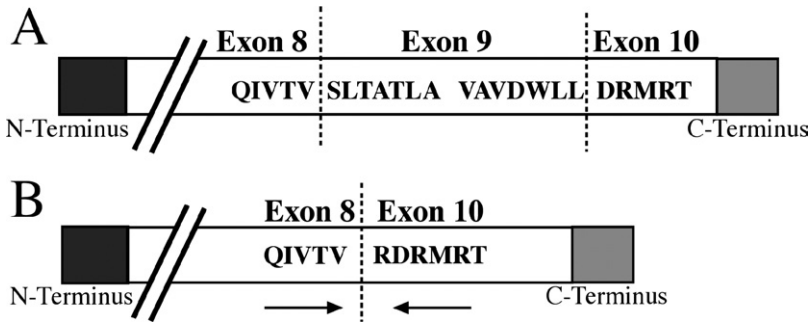

Fig. 1. Schematic diagram of the structure of EAAT2 and the peptides utilized in this study. (A) Normally spliced EAAT2. Peptide sequences straddling exons 8–9 and 9–10 correspond to peptides 2 and 3. (B) Exon 9–skipping EAAT2. The peptide sequence QIVTVRDRMRT (peptide 1) spans the exon 9 splice site.

(Fig. 1). The peptide was coupled to porcine thyroglobulin, (Sigma, Castle Hill, NSW, Australia).

Dot blots

To verify that the antibodies recognized the new splice site, peptide 1 was coupled to bovine serum albumin (BSA) for use in dot lots (see Pow et al., 2003 for details). To confirm that the antisera did not recognize the full-length form of EAAT2, two additional peptides H_2N-QIVTVSLTATLA and H_2N-VAVDWLLDRMRT (peptides 2 and 3) were also synthesized. These sequences represent the amino acid sequences overlapping the exons 8–9 and 9–10 boundaries respectively in the original sequence that includes exon 9 (Fig. 1). These peptides were similarly conjugated to BSA, for use in dot blots. To verify that the antiserum did not recognize the homologous exon 9 splice site in the closely related glutamate transporter EAAT3, the peptide H_2N-QIITIRDRFRT, representing the splice site region of that transporter was also synthesized and tested in dot blots.

Sera were tested by dot blotting (Pow et al., 2003) using peptides conjugated to BSA. One microliter of each conjugate was applied to PVDF membranes (Biorad, Sydney, Australia) and probed with the primary antisera or pre-immune sera at dilutions of 1:500–1:20,000. Detection was revealed using a biotinylated anti-rabbit secondary antibody and streptavidin–horseradish peroxidase complex (both from Amersham, Castle Hill, NSW Australia), with DAB as a chromogen. A BSA-biotin conjugate (40 ng) was also applied to each membrane as a positive control.

Western blotting

Brains from three adult Dark Agouti rats were collected after killing (sodium pentobarbital 100 mg/kg i.p.). Western blotting employed standard methods (Pow et al., 2003). Pre-absorption of antisera (50 μg of peptide one per ml of diluted antiserum) was used to confirm specificity of the antiserum. Conversely, pre-absorption with the other peptides tested by dot blotting was used to verify that staining persisted and was thus not attributable to either normal EAAT2, or to alternately spliced EAAT3.

Western blotting was carried out using standard methods. PVDF membranes were blocked using 5% BSA in Tris-buffered saline, then probed using the immune, pre-immune or pre-absorbed antiserum at a range of dilutions (1:500–1:50,000). Binding of primary antibodies was detected using the same methods as for dot blots.

Immunohistochemistry

Three rats from each of the age groups 7 days, 25 days or 12–14 weeks (young adults) were killed by overdose of sodium pentobarbital (100 mg/kg i.p.) and fixed by perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Tissues were dehydrated, embedded in paraffin wax and immunolabeled using standard immunoperoxidase or immunofluorescence techniques (Williams et al., 2005). Immunolabeling patterns for the exon 9–skipping form of EAAT2 were compared with those obtained using rat antibodies previously raised against GLT-1α. Controls included use of pre-immune serum and pre-absorption of dilute immune serum (50 μg of peptide/ml of diluted antiserum). In addition a commercial mouse monoclonal glial fibrillary acidic protein (GFAP) antibody (Sigma) was used to label a subset of astrocytes, particularly the GFAP immunoreactive types normally present in white matter in the adult brain.

RESULTS

Dot blotting

Initial screening by dot blotting demonstrated that the antiserum specifically recognized the peptide sequence that

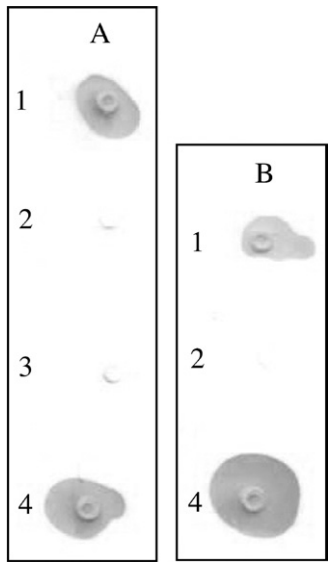

Fig. 2. Dot blots probed with the exon 9–skipping EAAT2 antibody. (A) Membranes dotted with 1 μl of conjugates of peptides 1–3 at positions 1–3 respectively. Biotinylated BSA was applied at position 4 as a positive control for the DAB reaction. (B) Blots of peptide 1 and the similar peptide for the exon 9–skipping form of EAAT3 at positions 1 and 2 respectively. The exon 9–skipping EAAT2 antibody is highly selective for exon 9–skipping EAAT2.