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Differential Regulation of 5′ Splice Variants of the Glutamate Transporter EAAT2 in an In Vivo Model of Chemical Hypoxia Induced by 3-Nitropropionic Acid

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Defective glutamate uptake has been implicated as a pathogenic event of neuronal damage related to cerebral ischemia and hypoxia. In several models of ischemia-hypoxia, a reduced immunoreactivity and altered RNA expression of excitatory amino acid transporter 2 (EAAT2), the major excitatory amino acid transporter, have been reported. However, the gene regulation of EAAT2 under these conditions is incompletely understood. In this study, we investigated alternative splicing of EAAT2 in an in vivo mouse model of chemical hypoxia as induced by 3-nitropropionic acid (3-NP). The neurotoxin 3-NP is an inhibitor of mitochondrial energy production. Furthermore, it is known to inhibit glutamate reuptake directly, representing at least one of the mechanisms responsible for 3-NP-induced neurodegeneration. Here we report an expression analysis of five known (mEAAT2/5UT1–5) and two novel (mEAAT2/5UT6, -7) 5′ splice variants of EAAT2 using semiquantitative PCR. The RNA expression was studied at 2, 12, 24, 48, and 72 hr and 7 days after 3-NP administration. mEAAT2/5UT4 and mEAAT2/5UT5 were up-regulated in the frontal cortex and down-regulated in the hippocampus 12–72 hr after chemical hypoxia. In the cerebellum, there was an increased expression of mEAAT2/5UT4 and a down-regulation of mEAAT2/5UT5. mEAAT2/5UT3 show a different regional expression pattern, being regulated in the cerebellum only. mEAAT2/5UT1–7 encoded distinct 5′ regulatory sequences, including conserved elements of translational control. It is easily conceivable that expression alterations of 5′ splice variants of EAAT2 are related to glutamate transporter malfunction after chemical hypoxia. Our findings contribute to the hypothesis that RNA splicing events can serve as a molecular mechanism of posthypoxic gene regulation.

Key words: glutamate; EAAT2; alternative splicing; hypoxia; 3-nitropropionic acid

The excitatory amino acid transporter 2 (EAAT2) is the main carrier of the neurotransmitter glutamate and mediates its rapid removal from the synaptic cleft. This process modulates the termination of glutamatergic synaptic signalling and serves to maintain extracellular glutamate below excitotoxic concentrations (Nicholls and Attwell, 1990). EAAT2 is an astroglial protein of transmembrane localization that is expressed predominantly in the cerebral cortex, the hippocampus, the caudate nucleus, the nucleus basalis of Meynert, and the spinal ventral horn, with lower levels of expression throughout the mammalian CNS (Milton et al., 1997). For several rodent models of transient ischemia and hypoxia, a diminished EAAT2 immunoreactivity and altered RNA expression have been reported (Torp et al., 1995; Martin et al., 1997; Bruhn et al., 2000; Fukamachi et al., 2001). Moreover, brain ischemia is known to induce a rapid reversal of EAAT2 function, with concomitant release of glutamate (Yamaguchi et al., 1998; Rossi et al., 2000). The compromised transporter expression has been implicated as a pathogenic event in posthypoxic neuronal damage.

Impairment of energy metabolism resulting from mitochondrial failure has been shown to play a major role in glutamate-induced cell death (Sanchez-Carbente and Massieu, 1999). The naturally occurring plant and fungal toxin 3-nitropropionic acid (3-NP) is an irreversible inhibitor of mitochondrial succinate dehydrogenase activity and a model substance with which to study hypoxic neuronal damage (Ludolph et al., 1992; Cavaliere et al., 2001). 3-NP at a low systemic dose (20 mg/kg) is known to

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TABLE I. Oligonucleotide Primers Used in PCR Amplification

Primer	Target, GenBank No.	Sequence	Position (nucleotides)
GSP1 (antisense)	EAAT2, AB007810	5′-GAAGGCTATCAA CATGACCACATC-3′	876–899
GSP2 (antisense)	EAAT2, AB007810	5′-CATGCGCATGCCCAGGTTTC-3′	751–770
CDS1 (sense)	EAAT2, AB007810	5′-GCTGTGGGCCTGCCAACG-3′	2,022–2,039
CDS2 (antisense)	EAAT2, AB007810	5′-GGTTCTTCCTCAACACTGCAG-3′	2,339–2,359
CDS3 (antisense)	EAAT2, AB007810	5′-GATTTCTAATGAGATCCAGGAAG-3′	1,160–1,182
5UT1 (sense)	mEAAT2/5UT1	5′-CATCAACAGAGGGTGCCAAC-3′	664–683
5UT2 (sense)	mEAAT2/5UT2	5′-CCCTGGCATTTAGGAGTTGC-3′	163–182
5UT3 (sense)	mEAAT2/5UT3	5′-GAGCCTTGATTCCGGATTCAG-3′	306–326
5UT4 (sense)	mEAAT2/5UT4	5′-TCCCATGACAGTATTGGAGTCAC-3′	80–102
5UT5 (sense)	mEAAT2/5UT5	5′-AGAACTTTGATCTCTGTGCC-3′	136–155
5UT6 (sense)	mEAAT2/5UT6	5′-CCTATCCCAGATGGGGACAG-3′	27–46
5UT7 (sense)	mEAAT2/5UT7	5′-GGGCTACTTCACAAATGGCAC-3′	59–79
S12s (sense)	S12, MMRPS12	5′-TCGCATCCAACTGTGATGAGCC-3′	211–232
S12as (antisense)	S12, MMRPS12	5′-CCTGAGATTCTTTGCCATAGTC-3′	393–414

induce a mild form of chemical hypoxia and does not result in pathological changes or behavioral symptoms in treated animals. The short metabolic impairment has been previously described as a preconditioning technique, which protects from a secondary lethal cerebral hypoxia (Wiegand et al., 1999). 3-NP is able to inhibit glutamate reuptake in a dose-dependent manner, representing at least one of the mechanisms responsible for the neurodegeneration in 3-NP-induced chemical hypoxia (Tavares et al., 2001).

Little is known about the gene regulation of glutamate transporters in ischemia/hypoxia and its experimental models, such as 3-NP-induced hypoxia (Gegelashvili and Schousboe, 1997; Sanchez-Carbente and Massieu, 1999). We found, under normal conditions in both the human and the mouse CNS, a complex posttranscriptional regulation of EAAT2, including alternative splicing of 5′ untranslated and coding sequences and differential cleavage/polyadenylation (Meyer et al., 1998; Münch et al., 2000, 2001, 2002). Furthermore, disease-related alterations of alternative splicing have been increasingly identified involving the abundance, location, or timing of normally expressed RNA isoforms (Daoud et al., 1999). However, it is unknown whether hypoxia affects the posttranscriptional gene regulation of EAAT2. In this study, we investigated splicing events of alternative EAAT2 transcripts encoding different 5′ regulatory sequences in a model of chemical hypoxia as induced by 3-NP. Here we report an expression analysis of five known (mEAAT2/5UT1–5) and two novel (mEAAT2/5UT6, -7) 5′ splice variants of EAAT2 in the 3-NP model.

MATERIALS AND METHODS

Molecular Cloning of Mouse EAAT2 Splice Variants

Two previously unknown transcripts of EAAT2 (mEAAT2/5UT6–7) were cloned from the normal mouse brain using 5′ rapid amplification of cDNA ends (5′-RACE). Murine brain cDNA was ligated with a 5′ adaptor sequence and subsequently polymerase chain reaction (PCR) amplified using adaptor primers (AP1, AP2; Clontech, Palo Alto, CA) and gene-specific primers (GSP1, GSP2; for primer positions, see Table I). The gene-specific primers were localized to highly conserved protein-coding sequences of EAAT2 in the 5′ part of the open reading frame. The primary PCR was performed with the primers AP1 and GSP1, followed by a secondary PCR amplification using the nested primers AP2 and GSP2. The PCR protocol for the primary and secondary reactions consisted of a two-step program of five cycles at 94°C (45 sec) and 70°C (4 min), followed by 25 cycles at 94°C (20 sec) and 68°C (4 min), respectively. The PCR products were subcloned (TA Cloning Kit; Invitrogen, Carlsbad, CA) and DNA sequenced (ABI 377 DNA Sequencer; Applied Biosystems, Foster City, CA). In situ hybridization (ISH) analysis of mEAAT2/5UT6 and mEAAT2/5UT7 was performed as described previously (Münch et al., 2002). In brief, brain sections (12 μm) were cut using a cryostat and kept frozen at –70°C until further processing. Radioactively labelled RNA probes were synthesized by in vitro transcription using SP6/T7 polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and ^{35}S-CTP (>1,000 Ci/mmol; Amersham, Piscataway, NJ). As a template, we constructed transcript-specific oligonucleotides encompassing the target sequence (see Fig. 1) and the SP6/T7 promoter sequence as follows: probe 1 complementary to nt 8–89 of mEAAT2/5UT6 and probe 2 nt 40–121 of mEAAT2/5UT7. Hybridization of the sections was carried out as previously described (Münch et al., 2002). Sections were rapidly dehydrated, dried, apposed to Kodak XAR-5 film, and developed after 3 weeks. The quantitative expression of mEAAT2/5UT6, -7 was further assessed in an RNA expression array of different mouse tissues (Multiple Tissue Expression Array; Clontech) that was hybridized with the RNA probes established during the ISH. The membranes were treated as previously described and exposed to Kodak XAR-5 film at –70°C for 24 hr (Münch et al., 2002).

Semiquantitative PCR

The quantitative expression of the EAAT2 splice variants mEAAT2/5UT1–7 was studied using semiquantitative RT-PCR. Total RNA was isolated from mouse frontal cortex, hippocampus, and cerebellum, which have topographical re-

mEAAT2/5UT6

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1                    GGGAGCC GCGCGCGGGG TGGTGCTGCC CTATCCCAGA TGGGGACA
48    GAACTGTGAC CCTGGACCCC TGAAACCCTA GCAGAGCGCT GGAGtgccaa caatatgccc
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mEAAT2/5UT7

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1                                                    CTCAGGCTT CCCGGAGC
20    AGAACAGAAT AAAAATAATA ACTAGGGACC CCAGCTTTTG GGCTACTTCA CAAATGGC
80    TAGCTGCTTT GGAGAGATCG GTGAACCTAC ATGTGACCTC AAAGtgccaa caatatgccc
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Fig. 1. Nucleotide sequence of the mouse EAAT2 splice variants mEAAT2/5UT6, -7 (5′ partial sequence). The translation start codon (ATG) is shown in boldface, and the exon boundaries are indicated by vertical lines. Lower-case letters indicate known 5′ sequences.

gions of good accessibility for tissue and RNA preparation (mRNA Isolation Kit; Applied Biosystems). One microgram was used as template for reverse transcription with random primers at 42°C for 50 min (RNA PCR Core Kit; Applied Biosystems). To reduce the potential influence of differential polyadenylation on reverse transcription and semiquantitative PCR, we performed reverse transcription of mRNA using random hexamers (Münch et al., 2000). For semiquantitative PCR, we designed primers specific for each of the mEAAT2/5UT1–7 transcripts (Table I). We performed a multiplex amplification of splice variant-specific and of two standard RNA sequences (mEAAT2/CDS, 40S ribosomal protein S12 RNA). mEAAT2/CDS represents a highly conserved part of the EAAT2 coding sequence accounting for the total amount of all known splice forms of the transporter RNA and was amplified using the primers CDS1 and CDS2. As an additional standard RNA, we coamplified the 40S ribosomal protein S12, a murine housekeeping gene with the primers S12s and S12as. The PCR was carried out using 10 ng of cDNA, 10 pmoles of each primer, and a program consisting of denaturation at 94°C (15 sec), annealing at 60°C (30 sec), and extension at 72°C (45 sec; AmpliTaq; Applied Biosystems). Five microliters of PCR products were removed after 28, 30, 32, 34, 36, 38, and 40 cycles; separated on a 2.5% agarose gel; and visualized by ethidium bromide staining. Images were captured by ultraviolet translumination (GelDoc1000; Bio-Rad, Hercules, CA) and quantified (MultiAnalyst software; Bio-Rad).

3-NP Model of Chemical Hypoxia

For the animal studies of chemically induced hypoxia, 3-NP was dissolved in 0.9% saline, and male CD1 mice (25–30 g) received a singular intraperitoneal injection of 20 mg/kg. None of the animals treated with 3-NP and used for the experiments showed symptoms of impaired motor or cognitive behavior. The mice were investigated at intervals of 2, 12, 24, 48, and 72 hr and 7 days (for each group n = 6) after the 3-NP administration and compared with nontreated controls (n = 6). The animal experimental protocols performed in this study conformed to the guidelines of the German Animal Care Act and were approved by the regional council of Tübingen and the ethics committee of the University of Ulm. Semiquantitative RT-PCR of splice variants mEAAT2/5UT1–7 was performed for tissue samples of the cerebral cortex, hippocampus, and cerebellum. The optical density values of PCR signals were quantified using an image-analysis system (MultiAnalyst software). Quantitative expression of EAAT2 transcripts was determined at cycle 38 during linear regression of amplification in relation to the internal standard S12 RNA. Statistical analysis of the data for six animals per time point was performed using Student's *t*-test and ANOVA with Fisher's protected *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

In this study, we investigated disease-related splicing events of 5′ splice variants of EAAT2 in a mouse model of chemical hypoxia. To achieve this aim, we searched for novel EAAT2 splice forms in the mouse brain and undertook an RNA expression analysis of all known transporter splice forms in the 3-NP model. 5′-RACE analysis revealed two novel 5′ variants of the mouse EAAT2 RNA, named *mEAAT2/5UT6* and *mEAAT2/5UT7* (Fig. 1). The transcripts were characterized by different 5′ untranslated sequences of 101 and 133 nucleotides, respectively, that show low homology to other 5′ variants of EAAT2 (Utsunomiya-Tate et al., 1997; Münch et al., 2002). The gross anatomical distributions of mEAAT2/5UT6 and mEAAT2/5UT7 were found to be similar as shown by ISH, which demonstrated the highest expression level in the neocortical areas, followed by the hippocampus > cerebellum > caudate nucleus. In other brain regions, only weak signals were detected, as presented exemplarily for mEAAT2/5UT6 in Figure 2. Quantitative expression analysis of multiple mouse tissues RNA arrays revealed a differential expression pattern in both neuronal and extraneuronal tissues (Fig. 2).

The relative expression of 5′ splice forms of EAAT2 (mEAAT2/5UT1–7) was studied at 2, 12, 24, 48, and 72 hr and 7 days after 3-NP administration and in nontreated control mice (Fig. 3). Six male CD1 mice were studied in each of the subgroups treated with 3-NP and in

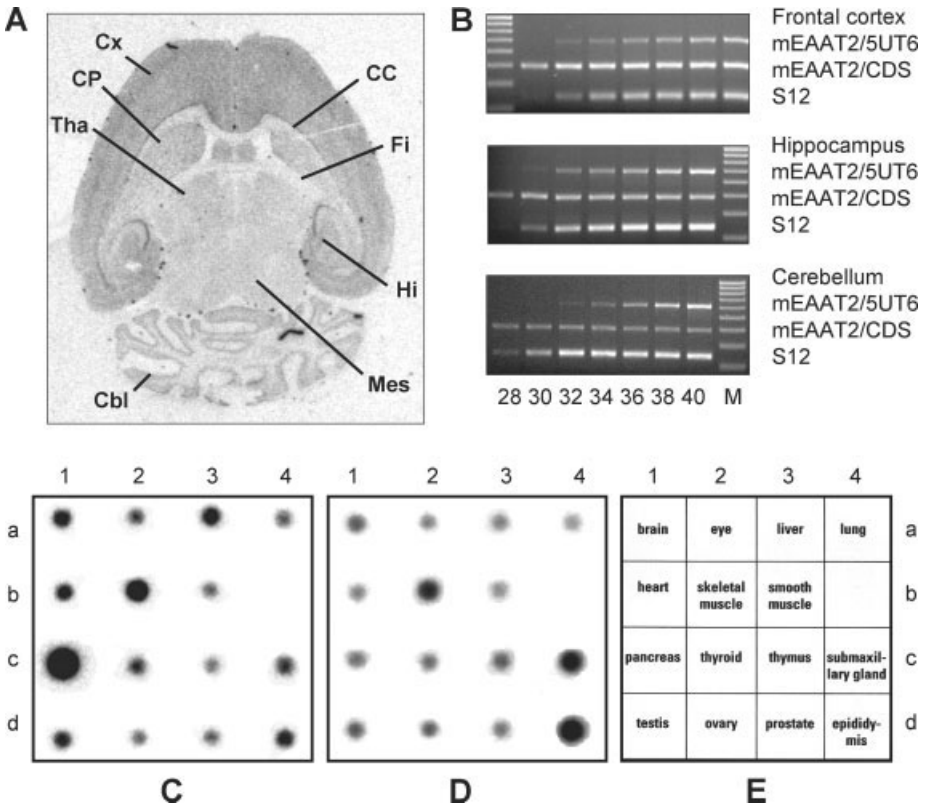

Fig. 2. **A:** In situ hybridization analysis of mEAAT2/UT6 in the normal mouse brain. The broad tissue distribution of mEAAT2/UT6 and mEAAT2/5UT7 was found to be similar and is exemplarily presented for mEAAT2/5UT6. **B:** Semiquantitative PCR analysis of mEAAT2/5UT6, the coding sequence of EAAT2 (mEAAT2/CDS), and the standard RNA (ribosomal protein S12) in the mouse frontal cortex, hippocampus, and cerebellum. PCR products obtained from cycle 28 to cycle 40 were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. **C–E:** RNA expression array of multiple mouse tissue specimens. mEAAT2/5UT6 (C) showed a moderate expression in the brain and abundant presence in the pancreas and skeletal muscle. mEAAT2/5UT7 (D) was prominently expressed in the epididymis > submaxillary gland > skeletal muscle. The legend for the multiple tissue array is given in E. Cbl, cerebellum; CC, corpus callosum; CP, caudate putamen; Cx, cortex; Fi, fimbria; Hi, hippocampus; M, molecular DNA marker; Mes, mesencephalon; Tha, thalamus.

the control group. Compared with the treatment group, the control animals showed the same genetic background, were grown in the same animal facility, and had been age matched. We identified a region-dependent and temporal expression pattern for three of the seven EAAT2 transcripts. Animals sacrificed after 2 hr revealed no significant alteration in the expression of EAAT2 transcripts. The first changes in the expression of EAAT2 RNA were observed 12 hr after 3-NP injection (Fig. 4). The effects of 3-NP on EAAT2 splice form regulation were found to continue for a maximum of 72 hr before returning to normal. The splice variants mEAAT2/5UT4 and mEAAT2/5UT5 showed a posthypoxic expression regulation in all regions of interest (hippocampus, cerebellum, frontal cortex). Both transcripts were transiently up-regulated in the frontal cortex, whereas a reduced expression of these transcripts was found in the hippocampus (12–72 hr post injection, $P < 0.05$). Interestingly, we identified in the cerebellum a counteracting expression pattern of these transcripts, showing a significantly increased expression of mEAAT2/5UT4 and a down-regulation of mEAAT2/5UT5 before returning to normal ($P < 0.05$, Fig. 4). mEAAT2/5UT3 showed a different regional expression pattern, being regulated in the cerebellum only ($P < 0.05$, Fig. 4). The posthypoxic regulation of transporter transcripts was confined to three 5′ splice variants of EAAT2 and was not observed for the constitutional transcript of EAAT2. Seven days after 3-NP administration, the relative expression of all EAAT2 transcripts had been normalized. The internal standard 40S ribosomal protein S12 RNA was invariably amplified in the series of brain tissues when equal amounts of total RNA were used as template for reverse transcription and subsequent semiquantitative PCR.

DISCUSSION

Alternative splicing is extensively used to create the structural and functional diversity of the mammalian brain (Smith and Valcarcel, 2000; Graveley, 2001). Differential splicing has been related to changes in ligand specificity (Miki et al., 1992), affinity (Davis et al., 1992), and electrophysiological properties of receptor proteins and neurotransporters (Hollmann et al., 1993). Posttranscriptional gene regulation in the brain is subject to developmental control and response to neuronal activity under normal and disease conditions (Grabowski and Black, 2001). It was the objective of this study to investigate the role of alternative splicing under the conditions of chemical hypoxia. We studied the splicing regulation of 5′ splice variants of the glutamate transporter EAAT2, for which a reduced protein expression has been found after ischemic-hypoxic insults (Torp et al., 1995). Furthermore, several hours after the induction of hypoxia, a substantial up-regulation of the EAAT2 RNA expression has been demonstrated, whereas reoxygenation normalized the expression levels of glutamate transporters (Hsu et al., 2001). Taken together, these studies support the view that alterations of transporter expression are centrally involved in

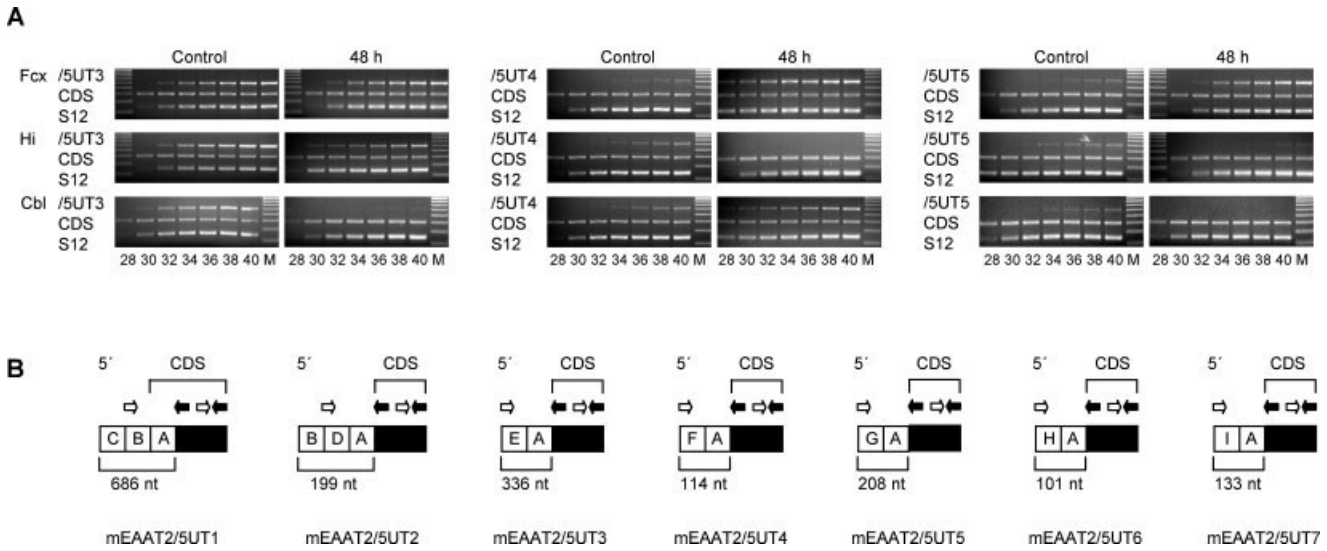

Fig. 3. **A:** Semiquantitative PCR analysis of 5′ splice variant sequences (mEAAT2/5UT3–5), the coding sequence of EAAT2 (CDS), and the standard RNA (ribosomal protein S12). Expression in the cerebral cortex (Fcx), hippocampus (Hi), and cerebellum (Cbl) is exemplarily shown at 48 hr (h) after the administration of 3-NP in the mouse model of chemical hypoxia and in nontreated controls. PCR products obtained at cycles 28–40 were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. **B:** Schematic representation of all known 5′ splice variants of the EAAT2 RNA (mEAAT2/5UT1–7). The 5′ exons resulting from alternative splicing are displayed in open boxes and are labelled A–I. The coding part (CDS) is symbolized by solid boxes. Arrows indicate the positions of primers used for PCR analysis. The nucleotide (nt) lengths of the 5′ partial sequences are based on GenBank AB007810 (mEAAT2/5UT1), AF528065 (mEAAT2/5UT2), AF528066 (mEAAT2/5UT3), AF528067 (mEAAT2/5UT4), and AF528068 (mEAAT2/5UT5) and on Figure 1 (mEAAT2/5UT6, -7).

the mechanisms of neurodegeneration in ischemia-hypoxia. Energy depletion during hypoxia has been found to be a determinant of hypoxic neuronal damage. The neurotoxin 3-NP causes the blockage of mitochondrial energy production that plays a major role in glutamate-induced cell death (Sanchez-Carbente and Massieu, 1999). The use of the 3-NP mouse model seems to be justified, insofar as an impairment of high-affinity glutamate uptake by 3-NP has been shown previously in rodent synaptosomes (Tavares et al., 2001). Apart from the participation of secondary excitotoxicity in the neurotoxic effects of 3-NP, direct involvement of the toxin in the glutamatergic neurotransmitter system has been described. In one report, 3-NP selectively prevented vesicular glutamate storage in a dose-dependent manner (Tavares et al., 2001). Furthermore, chronic treatment with 3-NP has led to a marked increase of neuronal susceptibility to glutamate uptake inhibition in vivo (Sanchez-Carbente and Massieu, 1999). However, little is known about the underlying mechanisms of transporter malfunction and the factors regulating EAAT2 gene expression in 3-NP-induced hypoxia.

We identified changes of quantitative expression of normally occurring EAAT2 splice forms that accompany 3-NP-induced neurotoxicity. Interestingly, the splice variant regulation was observed in a posthypoxic time interval between 12 and 72 hr and occurred in a region-dependent manner. This finding is in agreement with findings from rodent models of cerebral ischemia showing a prolonged down-regulation of the EAAT2 protein more than 24 hr after the ischemic insult (Torp et al., 1995). It has been previously described that the applied dose of 3-NP results in a low-grade decrease of succinate dehydrogenase activity, inhibition of the respiratory-chain energy production, and mild chemical hypoxia (Riepe et al., 1996). However, the 3-NP dose of 20 mg/kg used does not induce necrosis, apoptosis, or any other histologically detectable damage to the brain (Wiegand et al., 1999). The phenomenon of hypoxic-ischemic tolerance, in which preconditioning of the brain with mild hypoxia can induce tolerance to subsequent lethal hypoxia, is well known (Kirino et al., 1991). This tolerance can be induced by pretreatment with a variety of sublethal stresses, such as exposure to 3-NP. The mechanisms of ischemic-hypoxic preconditioning are not understood, although the involvement of various mechanisms of stress response and protection has been suggested. The alteration of glutamate transporter splicing and differential expression of EAAT2 splice variants in the mouse model of mild chemical hypoxia may point to a previously undescribed mechanism of preconditioning. However, this study, which was aimed at EAAT2 splicing in a model of mild hypoxia, has not addressed the role of EAAT2 in hypoxic tolerance. Further studies are required to investigate the involvement of glutamate transporter splicing in the mechanisms of chemical preconditioning.

The altered RNA expression of EAAT2 variants encoding different 5′ regulatory sequences may contribute

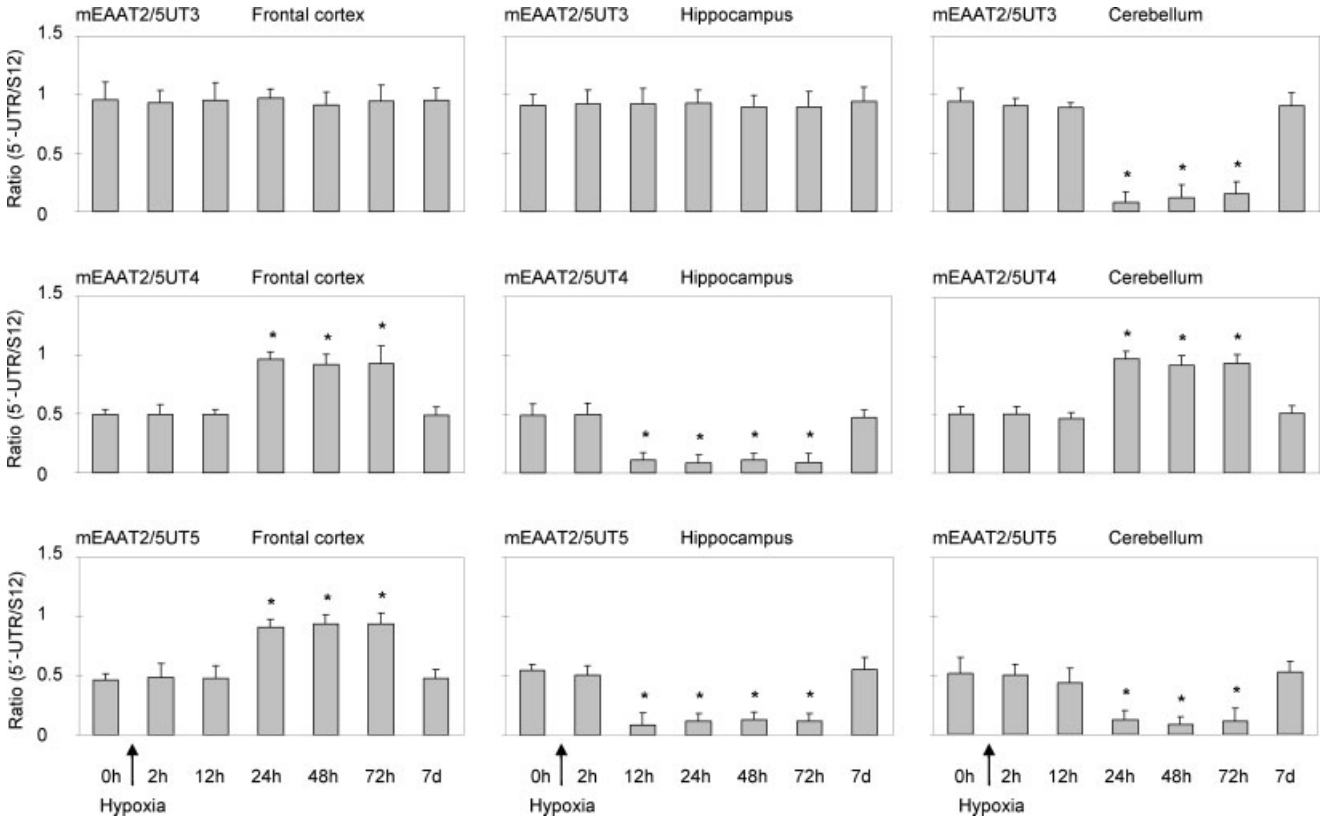

Fig. 4. Semiquantitative PCR analysis of mEAAT2/5UT3–5 in the cerebral cortex, hippocampus, and cerebellum of the mouse model of chemical hypoxia at 2, 12, 24, 48, and 72 hr (h) and 7 days (d) after the administration of 3-NP and compared with nontreated controls (0). The values represent relative expression levels of the EAAT2 splice variants compared with the expression of the standard RNA (S12). The results show the means (±SD) of six animals. $\star P < 0.05$ significantly different from the nontreated control group.

to defective glutamate uptake under hypoxic conditions. This hypothesis is supported by the presence of a single upstream open reading frame (uORF) in the transcript mEAAT2/5UT3 and three overlapping uORFs in mEAAT2/5UT5. In contrast, the transcript mEAAT2/5UT4 proved to be free of uAUG, which has been described in detail elsewhere (Münch et al., 2002). uORFs are one of the main structural features within the 5′ regulatory sequences of many stringently regulated messenger RNAs, including that of EAAT2 (Morris and Geballe, 2000). From other mRNAs, it is known that uAUGs followed by uORFs usually inhibit the translation from the major ORF (Morrish and Rumsby, 2001). Functional studies in these RNAs have revealed that the number of uAUGs inversely relates to translational efficacy (Arrick et al., 1991). However, the functional properties of the previously reported and novel 5′ regulatory sequences of EAAT2 splice variants are unknown as yet and remain to be delineated by detailed in vitro expression studies.

In summary, the mechanisms of posthypoxic glutamate transporter regulation are incompletely understood but may be associated with a time- and region-dependent expression of EAAT2 splice variants. It is readily conceivable that changing expression levels of EAAT2 5′ splice variants, as demonstrated in the mouse cortex, hippocampus, and cerebellum, are related to transporter malfunction after chemical hypoxia. Our findings contribute to the hypothesis that RNA splicing events may serve as an important molecular mechanism of posthypoxic gene regulation.

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