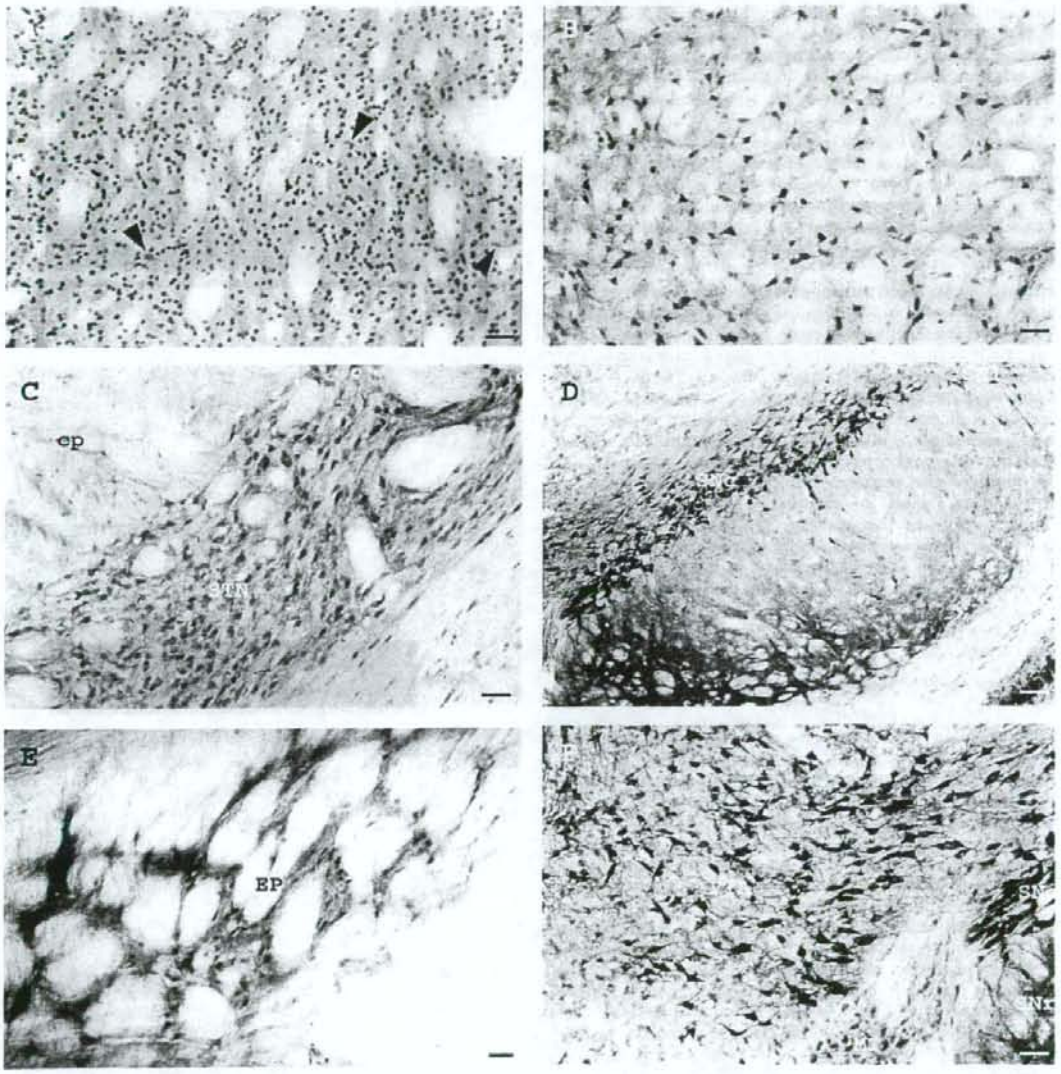

Fig. 6. Immunohistochemical staining in the presence of Triton X-100, illustrating the distribution of EAAT4 throughout the basal ganglia. **A:** Detail of the striatum. In the middle of a large number of homogeneously distributed intermediate-sized neurons, very few, faintly stained large neurons could be distinguished (arrowheads). **B,C:** Detail of the globus pallidus (B) and the subthalamic nucleus (STN; C). EAAT4-immunoreactive neurons were homogeneously distributed. EAAT4 label was present in the cell bodies and dendrites. **D:** A large number of intensely labeled neurons could be seen in the substantia nigra pars compacta (SNc). In the substantia nigra pars laterale (SNl) cells were less abundant but still intensely stained. Only a few intensely labeled cells were present in the substantia nigra pars reticulata (SNr), in the middle of a number of faintly stained neurons. **E:** In the entopeduncular nucleus (EP), sparse immunoreactive neurons could be visualized in a meshwork of immunoreactive neuropil. **F:** In the VTA intensely and homogeneously stained neurons were abundantly present. Abbreviations: cp, cerebral peduncle; MT, medial terminal nucleus accessory optic tract. Scale bar = 50 μm in A,B,D–F; 100 μm in C.

large, very faintly stained neurons could be detected when Triton X-100 was used (Fig. 6A, arrowhead). These large neurons probably represent the large aspiny cholinergic interneurons of type II (25–35 μm), which account for 1–2% of the total neuron population, because this is the only cell type in the striatum exhibiting this size (Yelnik, 2002). As in the

striatum, in the globus pallidus (Fig. 6B) and STN (Fig. 6C), EAAT4-immunoreactive neurons were homogeneously distributed. Staining was present in cell bodies and, contrary to the striatum, also in dendrites. In the EP, a few immunoreactive neurons could be visualized, whereas neuropil staining was moderate (Fig. 6E).

As for the SNc (Figs. 2D, 6D) and VTA (Figs. 2B,C, 6F), very intensely and homogeneously stained, densely packed neurons were visualized after EAAT4 staining. The nucleus of some immunopositive neurons was devoid of this intense and homogeneous labeling, yet was often covered by a number of immunoreactive puncta (Fig. 2C, arrow). Still other neurons and most of the dendrites were defined only by these immunoreactive puncta (Fig. 2D,E). In the substantia nigra pars laterale (SNl), the staining pattern of the immunoreactive neurons was similar to that of the SNc; however, the density of immunoreactive neurons was significantly smaller compared with the SNc. In contrast, in the substantia nigra pars reticulata (SNr), an occasional intensely stained neuron was present in the middle of a small number of faintly stained neurons, characterized by a punctate labeling (Fig. 6D).

Mesencephalic regions. EAAT4 was omnipresent in the midbrain region. Besides the aforementioned high expression levels in the IP (Fig. 5F) and VTA (Fig. 6F), EAAT4 was also enriched in the superficial layers of the SC (Fig. 4A). The immunoreactive signal in the SC was higher than average, with a very intense neuropil staining in the zonal layer and superficial gray layer. In the optic nerve layer, cell bodies as well as dendrites showed clear EAAT4 labeling.

Reverse transcription polymerase chain reaction

The presence of EAAT4 in the fasciculus retroflexus was further investigated on the mRNA level, given the unexpected abundant occurrence of EAAT4 protein in an axon bundle. After collecting the tissue samples by means of the LMD technique (Fig. 7A–C), making it possible to isolate tissue very precisely from the fasciculus retroflexus without contamination from any other nearby brain tissue, RT-PCR was performed with up- and downstream primers corresponding to rat EAAT4 nucleotide sequences 906–926 and 1,299–1,319, respectively (Lin et al., 1998; Massie et al., 2001). As a control we also included mRNA samples of the cerebellum, cerebral cortex, striatum, and hippocampus. For each condition a fragment was amplified with a length of 414 bp, the expected length based on the known sequence (Fig. 7D). No fragment was amplified when the cDNA in the PCR reaction mixture was replaced by water.

Real-time PCR

In order to estimate tissue expression levels of EAAT4 mRNA, semiquantitative analysis was performed by using real-time PCR. As expected, the cerebellum was found to contain by far the highest levels of EAAT4 mRNA (Fig. 7E). By comparison, after setting the cerebellum as calibrator, the cerebral cortex contained 6.7 ± 1.8%, the hippocampus 2.0 ± 0.4%, the striatum 1.6 ± 0.2%, and the fasciculus retroflexus 4.7 ± 1.8% of the total cerebellar EAAT4 mRNA content (n = 3). These mRNA levels are on the same order of magnitude as those measured by Ward et al. (2004) in the cerebral cortex, i.e. 3.1% relative to the cerebellum.

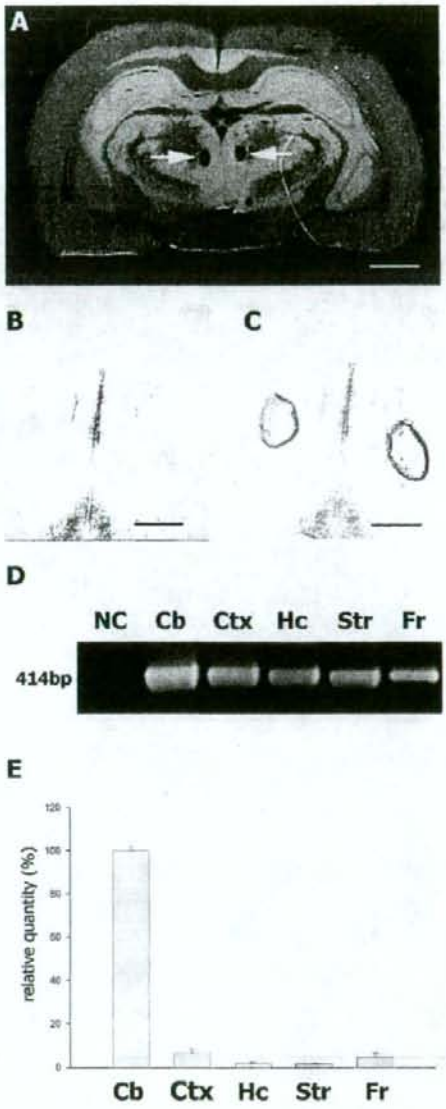

Fig. 7. **A:** Photograph showing a frontal section from which the fasciculus retroflexus (arrows) was bilaterally removed by laser microdissection. **B,C:** Detail of the same section before (B) and after (C) the fasciculus retroflexus was laser-collected. **D:** Conventional end-point RT-PCR with specific primers for EAAT4 on mRNA from the cerebellum (Cb), cerebral cortex (Ctx), hippocampus (Hc), striatum (Str), and laser-captured fasciculus retroflexus (Fr). No band could be detected in the negative control lane (NC), whereas for all other conditions a band of 414 bp was visualized. **E:** Real-time PCR analysis of EAAT4 mRNA expression in rat cerebellum (Cb), cerebral cortex (Ctx), hippocampus (Hc), striatum (Str), and fasciculus retroflexus (Fr). EAAT4 mRNA expression levels were normalized to GAPDH. The amount of transcript in forebrain regions was expressed as mean value (n = 3, SD) relative to cerebellum (= 100%). Scale bar = 2 mm in A; 0.5 mm in B,C.

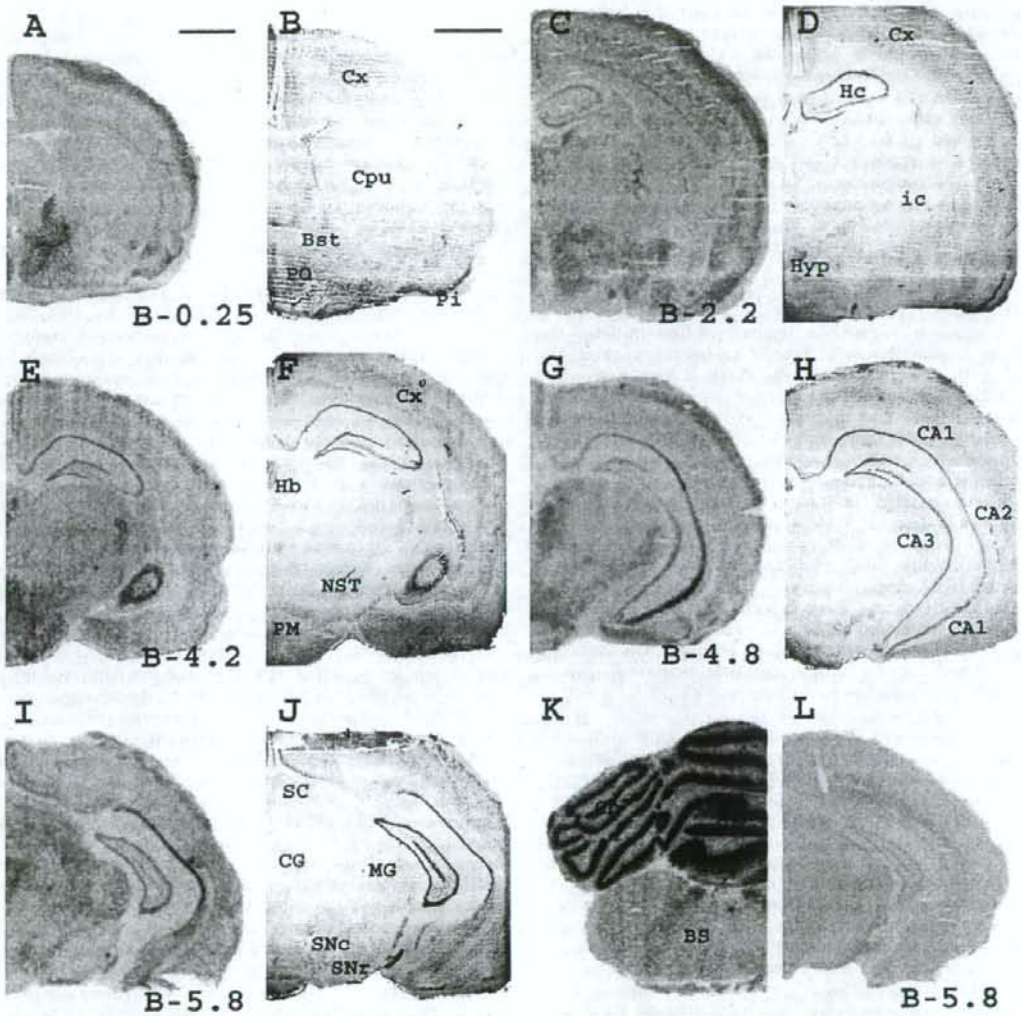

Fig. 8. Distribution of EAAT4 mRNA throughout the fore- and midbrain (A–J) and cerebellum (K). Autoradiograms were generated after in situ hybridization by using EAAT4 ^{35}S-labeled antisense (A,C,E,G,I,K) and sense (L) riboprobes. Neighboring sections were stained with Richardson's methylene blue-azure II Nissl stain (B,D, F,H,J). **A–J:** In the fore -and midbrain, labeling was higher than average in layers II/III and layer V of the cerebral cortex (Cx; A,C,E,G), the substantia nigra pars compacta (SNc; I), the medial geniculate nucleus (MG; J), and the superior colliculus (SC; J). Clear EAAT4 labeling was observed in the preotpic area (PO; A), the bed nucleus of the stria terminalis (Bst; A), piriform cortex (Pi; A), hypothalamic region (Hyp; C), premammillary nuclei (PM; E), the CA1 region of the hippocampus (Hc; G), and the central gray (CG; J). K: A very high signal was observed in the cerebellum (Cb). Abbreviations: BS, brainstem; CPu, caudate putamen; Hb, habenular nuclei; ic, internal capsule; NST, subthalamic nucleus; SNr, substantia nigra pars reticulate. Scale bar 2 mm. Scale bar in panel A applies to all in situ hybridization figures (i.e. panel C, E, G, I, K, L), scale bar in B applies to all Nissl stains (i.e. D, F, H, J).

In situ hybridization

As a last verification of the immunohistochemical data for EAAT4, in situ hybridization was performed on sections containing some important brain regions (Fig. 8). EAAT4 mRNA expression was by far highest in the cerebellar cortex (Fig. 8K). As for the cerebral cortex, we could clearly distinguish a layered pattern with the highest signal in layers II/III followed by layer V (Fig. 8A,C,E,G,I). Also, a relatively intense signal was observed in the piri-

form cortex (Fig. 8A,C). As for the hippocampal formation, relatively strong labeling could be seen in all CA regions, with the most intense signal in CA1 and in the dentate gyrus (Fig. 8E,G). Concerning the nuclei of the basal ganglia, high EAAT4 signal was present in the SNc (Fig. 8I). In addition, clear EAAT4 labeling occurred in the preoptic area, the bed nucleus of the stria terminalis, several hypothalamic, amygdaloid, and premammillary nuclei (Fig. 8C,E), in the central gray, and in the intermediate gray layer of the superior colliculus (Fig. 8I). Labeling with the sense probe resulted in a faint background signal (Fig. 8L).

DISCUSSION

In this paper we describe for the first time in detail the widespread distribution of EAAT4 protein throughout the rat fore- and midbrain. EAAT4, which is highly enriched in the Purkinje cells of the cerebellum, was omnipresent in the rat fore- and midbrain, albeit at protein levels significantly lower compared with those in the cerebellum. On the whole, EAAT4-IR was localized not merely in glutamatergic and GABAergic neurons, but also in dopaminergic and probably cholinergic neurons. Besides the neuronal localization of EAAT4 protein, a very faint glial labeling in white matter of several CNS regions as well as in the ventricular walls could be observed. Given the unexpected high expression level of EAAT4 protein in the fasciculus retroflexus, the presence of EAAT4 in this axon bundle was confirmed at the mRNA level and estimated to be 4.7% that of the cerebellum. In addition, the distribution of EAAT4 in the main brain nuclei was confirmed on the mRNA level by using conventional RT-PCR as well as in situ hybridization.

In general, given the presence of the glial glutamate transporters, which are, in most fore- and midbrain regions, responsible for the bulk of glutamate reuptake, and given the low glutamate transport rate of EAAT4 (Torres-Salazar and Fahlke, 2007), we can imagine that the functional significance of EAAT4 in all these regions is not solely linked to glutamate reuptake activity. EAAT4 has large substrate-gated Cl^- currents that are not coupled to substrate transport. Thus, besides taking up glutamate to terminate glutamate neurotransmission, EAAT4 might also modulate neurotransmission by dampening of neuronal excitability via the substrate-gated anion conductance, without interfering with glutamate homeostasis. In addition, it has been noted that metabotropic glutamate receptor activation is specifically controlled by neuronal glutamate transporters in the cerebellar cortex (Brasnjo and Otis, 2001), and it was suggested by Otis et al. (2004) that this interaction could influence synaptic plasticity in a synapse-specific manner. Moreover, metabotropic glutamate receptors and neuronal glutamate transporters, which are closely associated in the perisynaptic space, can serve together as a physiological mechanism for limiting glutamate spillover from excitatory synapses (Otis et al., 2004). This is further supported by results obtained from EAAT4-deficient mice, indicating that indeed in the cerebellum EAAT4 is responsible for effectively preventing glutamate from spilling over to neighboring synapses (Takayasu et al., 2005).

Unfortunately, all studies on mice lacking EAAT4 are uninformative on brain regions outside the cerebellum (Huang et al., 2004; Takayasu et al., 2005, Yamashita et al., 2006). The reuptake of glutamate by EAAT4 can also have a metabolic role. After being transported into the cell, glutamate can be converted into α-ketoglutarate by glutamic acid decarboxylase and then enter the tricarboxylic acid cycle to produce ATP. The functionality of EAAT4 in the fore- and midbrain is further supported by the observation of Huerta et al. (2006) that the mRNA of the EAAT4-associated interacting proteins KIAA0302 and ARHGEF11 is highly expressed throughout the brain.

In the hippocampal formation, a strong somatodendritic labeling could be observed in the pyramidal cell layer of the subiculum. Also, the pyramidal cell layer of CA1–3 and the granular cell layer of the dentate gyrus showed a staining higher than average. Besides EAAT4, all other glutamate transporter subtypes (Lehre et al., 1995; Kugler and Schmitt, 1999) as well as glutamate receptor subtypes (Monaghan et al., 1989; Petralia and Wenthold, 1992) are expressed throughout the hippocampus, which is not surprising given that the glutamatergic as well as the GABAergic in- and output to all parts of the hippocampal formation is quite abundant (Ottersen and Storm-Mathisen, 1984). Moreover, in the stratum radiatum of CA1, synapses are often found side by side without any intervening glial processes (Harris and Stevens, 1989; Sorra and Harris, 1993; Lehre and Danbolt, 1998), making neuronal glutamate reuptake more important relative to other brain regions (Rothstein et al., 1996).

Concerning the nuclei of the basal ganglia, very strong staining could be observed in the SNc and in the VTA, a basal ganglia-related structure. Neurons from the SNc receive, among other inputs, glutamatergic input from the medial prefrontal cortex, the STN, and the pedunculopontine region. Also the VTA receives glutamatergic input from a number of different brain structures, including the prefrontal cortex (Carr and Sesack, 2000; Sesack and Pickel, 1992; Thierry et al., 1983), the pedunculopontine nucleus (Charara et al., 1996; Kelland et al., 1993), and the bed nucleus of the stria terminalis (Georges and Aston-Jones, 2001, 2002). In the SNc as well as the VTA, EAAT4 is present on dopaminergic neurons. Direct evidence comes from staining performed on rats with 6-OHDA lesions of the medial forebrain bundle. Five weeks after lesioning, a dopaminergic cell loss of 90% can be observed in the SN and VTA (Sarre et al., 2004), which corresponds to the loss of EAAT4-immunoreactive cells that we observe in both nuclei (personal observations). The glutamatergic afferents to the SNc and VTA are probably involved in the regulation of these dopaminergic neurons. Therefore, several glutamate receptor subtypes (*N*-methyl-D-aspartate [NMDA] and non-NMDA) have been found in both brain regions (Fallon and Loughlin, 1995; Kalivas, 1993). For the same reasons it is not surprising that EAAT4 has a high expression level in these neurons.

Regarding the other nuclei of the basal ganglia, as discussed above, we detected a relatively high expression level of EAAT4 in the striatum as well as the STN. Interestingly, both nuclei share common characteristics because they both receive cortical and thalamic afferents (Parent, 1986; Canteras et al., 1990) and project to the pallidum and SN (Parent, 1986; Kita and Kitai, 1987). In GABAergic cells, glutamate taken up by EAAT4 can serve as a precursor for neosynthesis of GABA (Furuta et al., 1997; Seal and Amara, 1999) and thus enhance the inhibitory synaptic strength. In addition, the presence of EAAT4 on striatal neurons might be part of the glutama-

tergic regulation of the dopaminergic activity in the striatum as well as the STN, described by Wüllner et al. (1994) and Ampe et al. (2007), respectively. Small fractions of ionotropic and metabotropic striatal EAA binding sites are located on dopaminergic terminals where they may have a distinct impact on dopaminergic activity. As for the expression of EAAT4 on cholinergic neurons, we might speculate that, again, it is linked to its chloride channel properties, which make it behave, to some extent, as an inhibitory glutamate receptor (Dehnes et al., 1998). In addition, as described above, EAAT4 can transport glutamate into the neuron, which can then serve as an energy source.

A moderate expression level could be observed in the globus pallidus and the EP. In the SNr only very few intensely labeled neurons could be seen intermingled with a moderate number of faintly stained neurons. However, in addition to the GABAergic input provided by the caudate-putamen (Chevalier and Deniau, 1990; Deniau et al., 1978), a prominent glutamatergic innervation of the SNr is provided by fibers of the STN (Hammond et al., 1978; Kitai and Kita, 1987; Nakanishi et al., 1987), and all classes of glutamate receptor subtypes are present in this area (Albin et al., 1992).

EAAT4-IR was very pronounced in the habenulo-interpeduncular system, including the fasciculus retroflexus. Given this unexpected expression of EAAT4 in an axon bundle, we further investigated the presence of EAAT4 here. Real-time PCR revealed a relatively high amount of EAAT4 mRNA in the fasciculus retroflexus compared with the other fore- and midbrain regions examined. Surprisingly, and in sharp contrast to EAAT4, for all other high-affinity glutamate transporters, i.e., GLAST, GLT-1, and EAAC1, the fasciculus retroflexus is devoid of immunolabeling. In addition, the glial glutamate transporters GLAST and GLT-1 are absent from the MHb, whereas EAAC1, like EAAT4, is expressed in this nucleus. However, all glutamate transporters show a considerable expression level in the LHb (personal observations). The MHb contains cholinergic and substance P-containing neurons, the former being crowded in the ventral two-thirds of the nucleus whereas the latter are exclusively localized in the dorsal part (Contestabile et al., 1987). Some neurons of the MHb feature dense glutamatergic innervation (Robertson et al., 1999), and glutamate serves as the excitatory transmitter at MHb-IP synapses (Brown et al., 1983; McGehee et al., 1995). This might explain the very high expression levels of EAAT4 in both aforementioned nuclei as well as the expression of metabotropic glutamate receptors, as reported before by Kinoshita et al. (1998).

The fasciculus retroflexus however, is an axonal tract. Projecting GABAergic neurons from the LHb sent axons through the mantle of the tract to midbrain cell targets (the SN, VTA, and raphe) (Herkenham and Nauta, 1979; Carlson et al., 2000). In contrast, the MHb projects through the core of the tract, corresponding to the cholinergic half of the fasciculus retroflexus (Herkenham and Nauta, 1979; Woolf and Butcher, 1989), to the IP. This part of the fasciculus retroflexus contains the highest concentration of nicotinic receptors in brain (London et al., 1985; Perry and Kellar, 1995). Our staining suggests that the immunoreactive fibers originate in the LHb as well as the MHb. These fibers could be followed until arrival in the IP. We were able to detect branching of the fasciculus retroflexus only once, which is not surprising given the small width of such branches.

The most plausible explanation for the labeling of this axon bundle with the EAAT4 antibodies is that not the axons but the glial processes, which are intimately associated with the axons, contain EAAT4 protein and mRNA.

The faint glial labeling obtained with the EAAT4 antiserum was not restricted to glial cells located in the white matter of several CNS regions. Also, ependymal cells lining the lateral and third ventricle were stained. Hu et al. (2003) detected glial labeling. However, they did not observe colocalization of EAAT4-IR with an oligodendrocyte marker, whereas they did with an astrocyte marker. Our data do not exclude the presence of EAAT4 in astrocytes, although the typical arrangement of the majority of the cells in rows strongly suggests that EAAT4 protein is localized to oligodendrocytes. Also, EAAC1, originally considered to be confined to neurons, was localized to glial cells. In accordance with our data, EAAC1 was expressed in oligodendrocytes of white matter, in ependymal cells, and in epithelial cells of the choroid plexus (Kugler and Schmitt, 1999). In epithelial cells of the choroid plexus no GLAST or GLT-1 could be detected, contrary to the tanycytes and ependymal cells (Berger and Hediger, 2000, 2001). Therefore, the presence of EAAC1 and EAAT4 in the choroid plexus might be important to prevent the passage of glutamate from the blood stream into the cerebrospinal fluid, where the glutamate concentration is very low, as stated by Kugler and Schmitt (1999).

In conclusion, some areas of high EAAT4-IR coincide with target areas of dense glutamatergic innervation, e.g., some corticofugal pathways, as described before for GLAST and GLT-1 (Lehre et al., 1995). However, some areas known to be low in glutamatergic innervation, e.g. globus pallidus, also show a considerable EAAT4 expression level, indeed suggesting that the role of EAAT4 in these regions goes beyond the canonical role of glutamate removal. Thus, whether the functional significance of this widespread distribution of EAAT4 in the fore- and midbrain is related to its re-uptake activities or to possible other functional roles that this transporter can play, on account of its chloride channel properties (Sonders and Amara, 1996; Seal and Amara, 1999) or its close association with metabotropic glutamate receptors (Otis et al., 2004), needs further investigation. After all, besides decreasing the total glutamate concentrations, EAAT4 can also prevent excessive excitation and help membrane repolarization inasmuch as its activation elicits chloride influx and consequent local hyperpolarization (Raiteri et al., 2002). Moreover, the interaction of the neuronal glutamate transporters with the metabotropic glutamate receptors can influence synaptic plasticity as well as limit the glutamate spillover from excitatory synapses, as described for the cerebellum (Otis et al., 2004).

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Edited GluR2, a gatekeeper for motor neurone survival?

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Summary

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disorder of motor neurones. Although the genetic basis of familial forms of ALS has been well explored, the molecular basis of sporadic ALS is less well understood. Recent evidence has linked sporadic ALS with the failure to edit key residues in ionotropic glutamate receptors, resulting in excessive influx of calcium ions into motor neurones which in turn triggers cell death. Here we suggest that edited AMPA glutamate (GluR2) receptor subunits serve as gatekeepers for motor neurone survival. *BioEssays* **30:1185–1192, 2008.**

Introduction

Amyotrophic lateral sclerosis (ALS), sometimes referred to as Lou-Gehrig's disease after the renowned American baseball player who suffered from the condition, is a progressive disorder of motor neurones, characterized by both pyramidal tract symptoms of spasticity with pathological reflexes, as well as lower motor neurone defects leading to progressive muscle wasting and inevitable death from respiratory muscle paralysis within a few years of onset (Fig. 1). The pathological hallmark of ALS is selective vulnerability of motor neurons, which show neuronal inclusion bodies including Bunina bodies and ubiquitinated Lewy-like or skein-like formations (Fig. 1). These structures are found in most patients with sporadic ALS but not in those with familial ALS associated with SOD1 mutations. Some cases of ALS are inherited, and may even represent multiple disease types (Table 1), but the majority (95%) are sporadic, with poorly understood aetiology, as none of the genes that cause familial ALS have so far been shown to be associated with sporadic ALS. The molecular mechanisms that underlie selective degeneration of motor neurones while sparing other neuronal cell types, including other neurons within the dorsal horn, are unresolved. Among several possible explanations, excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subtype of ionotropic glutamate receptors, has attracted much attention due to the fact that motor neurons are particularly vulnerable to AMPA receptor-mediated neurotoxicity *in vivo* as well as in cultured spinal cord neurons.[1]

Ionotropic receptors for glutamate (iGluRs) are important in mediating fast glutamatergic synaptic transmission in the vertebrate nervous system. Three distinct iGluR families were initially defined by their sensitivity to ligands:[2] the N-methyl-D-aspartate (NMDA type), the (S)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazole) propionic acid (AMPA type) and the kainate type. Human iGluRs are composed of the products of seven NMDA receptor subunit genes (NR1, NR2A–D, NR3A and NR3B), four AMPA receptor genes (GluR1–GluR4) and five kainate receptor genes (GluR5–GluR7, plus KA1 and KA2). Co-assembly of subunit within families gives rise to a large number of receptor subtypes with distinct pharmacological and physiological properties. An increased influx of Ca^{2+} through activated AMPA receptors, which is regulated by the presence or absence of the Q/R editing at a site within the pore region of the GluR2 subunit (the unedited form is highly permeable to Ca^{2+}), plays a key role in slow death of motor neurons in culture (Fig. 2). In this context, an exciting new avenue for research has emerged with the discovery that sporadic ALS patients have a defect in pre-mRNA editing of the ionotropic glutamate (AMPA) receptor subunit, GluR2. Editing of this receptor is developmentally controlled and failure to edit results in motor neurone loss and early death of the organism. Here we discuss the significance of the under-editing phenotype as a characteristic of the sporadic forms of ALS.

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Abbreviations: ALS: amyotropic lateral sclerosis; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; iGluRs: ionotropic receptors for glutamate; NMDA: N-methyl-D-aspartate; ADARs: deaminases acting on RNA; nAChRs: nicotinic acetylcholine receptors; SBMA: spinal and bulbar muscular atrophy; DRPLA: dentatorubral–pallido–luysian atrophy; SBMA: spinal and bulbar muscular atrophy; DRG: dorsal root ganglion; EAAT2: glutamate transporter of astroglia; MSA: multiple system atrophy; LGIC: ligand-gated ion channel; MN: motor neurone; UMN: upper motor neurone.

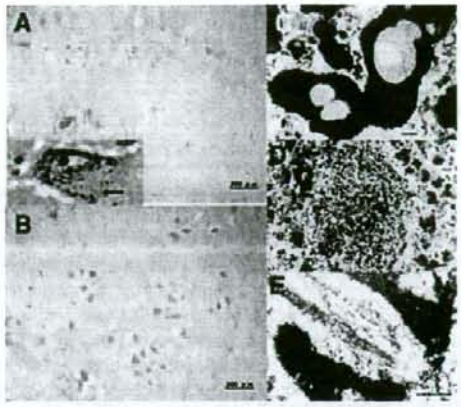

Figure 1. Neuropathological characteristics of sporadic ALS. **A:** There is a marked loss in the number of large neurons in the anterior horn of the spinal cord of a sporadic ALS case as compared to a control subject as shown in **B**. Some remaining motor neurons show bare characteristic cytoplasmic inclusion bodies, including the Bunina body (inset and **C**), Lewy-like inclusion (round body) (**D**) and skein-like inclusion (**E**). These inclusions are the pathological hallmark of sporadic ALS. Bars are 1 μm (**C, E**) and 5 μm (**D**). (Courtesy of Professor Shoichi Sasaki at the Tokyo Women's Medical University).

RNA editing of AMPA-type glutamate receptors and other ligand-gated ion channels adds to their functional diversity

The repertoire of iGluRs is expanded further by alternative splicing, which can affect various receptor properties such as their pharmacological characteristics,[3] desensitization (e.g. AMPA receptor "flip" and "flop" variants[4]), interaction with other proteins (e.g. GluR6a and GluR6b[5]) and trafficking (e.g. GluR7a and GluR7b[6]). Diversification of iGluRs is also increased by RNA A-to-I editing in which "deaminases acting on RNA" (ADARs)[7] cause selected adenosine residues in the genome to be read as guanosine in transcripts by converting an adenosine to an inosine[7,8] (Fig. 3). There are three human ADAR members, of which ADAR2 plays a major role in GluR2 Q/R site-editing, whereas other editing positions are considered to be catalyzed by either ADAR1 or ADAR2. Three AMPA (GluR2, GluR3 and GluR4) and two Kainate receptors (GluR5 and GluR6) are known to undergo RNA editing[9,10] which alters amino acid residues in functionally significant regions (Table 2).

Editing is not restricted to GluRs; RNA editing is also seen in nicotinic acetylcholine receptors (nAChRs) of Drosophila, the first organism for which RNA editing of nAChRs was demonstrated, with editing being shown for the three α subunits, Dα5, Dα6 and Dα7.[11] This finding was confirmed and extended in a comparative genomics approach, in which Hoopengardner and colleagues identified 16 ADAR targets in Drosophila, including additional nAChR subunits, Dβ1, Dβ2 as well as the ionotropic GABA receptor (GABAR) subunit, RDL[12,13] and a glutamate-gated nAChR chloride channel.[14] The RNA editing sites in the nicotinic acetylcholine receptor subunits[15] are present in the transmembrane region and ligand-binding domains, so they might potentially affect channel function, although difficulties in expressing Drosophila nAChR subunits has so far prevented this from being tested experimentally. Interestingly, the editing sites in nAChR subunits of different insects are only partially conserved, giving rise to species-specific isoforms.[16] RNA editing of the rat α3 glycine receptor (GlyR) enhances agonist potency.[17] In mice, editing of the α3 GABA receptor subunit is developmentally regulated and affects activation and deactivation kinetics and rectification.[18] In humans, however, editing for LGICs other than glutamate or GABA has not been demonstrated, although there is editing in a G-protein coupled receptor, 5-HT2C, with possible links to depression.[19] The editing of human ionotropic glutamate receptors may also be of important functional significance.

RNA editing at the Q/R site of the AMPA receptor GluR2 subunit reduces calcium permeability and protects neurones

The Q/R switch at amino acid 607 in the second transmembrane domain of GluR2 is attributable to RNA editing.[20] GluR2-containing AMPA receptors are normally impermeable to calcium ions because of editing at this site. Consequently, failure to edit the Q/R site results in a channel permeable to calcium ions. Thus, AMPA receptors that contain unedited GluR2 (or lack GluR2 altogether) are permeable to calcium and there is abundant evidence, particularly from studies on ischemia, that this calcium permeability renders neurones vulnerable to excitotoxic cell death. First, there is a time delay between overstimulation of AMPARs and resulting cell death. For example, the observation that, following transient global ischemia, neurodegeneration does not occur until 48–72 hours after circulation has been restored[21,22] is consistent with cell death being caused by an excessive accumulation of intracellular calcium resulting from overstimulation of calcium-permeable glutamate receptors.[23] Secondly, ischemic cell death appears to depend upon increased calcium influx through AMPARs. For instance, in animal models of ischemia and epilepsy, it has been confirmed that before vulnerable neurones die, GluR2 subunit expression is down-regulated and this is accompanied by an enhanced calcium component in their excitatory postsynaptic potentials.[24] Furthermore, antisense oligonucleotides to GluR2 enhanced neuronal death and ischemic pathogenicity[24,25] and over-expression of Ca-permeable AMPARs promotes ischemic cell

Table 1. Familial forms of amyotrophic lateral sclerosis (ALS) have been categorized into 8 types according to the locus of mutation (where known) and the age of onset and progression of the disease.

ALS type	Gene	onset	cells
1	SOD1 NEFH	Adult (40–60 yo)	Anterior horn Autosomal dominant 3% of cases of sporadic ALS[1]
2	ALS2[2]	Juvenile (3–20)	Autosomal recessive UMNs of pyramidal tract Results from short-form splice variant of ALS2[3]
3	unknown	Adult (45)	Anterior horn
4	SETX[4] senataxin: RNA processing	Juvenile (<25)	Autosomal dominant No bulbar involvement Long duration – some with full lifespan slowly progressive distal muscle weakness and atrophy with UMN signs, normal sensation, and absence of bulbar involvement [5]
5	Linked to 15q15-q22[6]	8–18	Autosomal recessive Upper mn and lower mn signs, fasciculation
6	Linkage to 16q12[7] or 9p 13.2–21.3[8]	37–66	Limb onset, bulbar involvement
7	unknown		Clinical presentation not reported
8	VAPB TDP-43	Adult (25–44 yo) Adult (50–70 yo)	Autosomal dominant Spinal or bulbar onset[9–11]

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death.[26] This suggests that a reduction in GluR2-containing receptors is causative of neuronal death in ischemia. Thus, RNA editing may play a key role in preventing cell death through regulating the calcium permeability of ionotropic glutamate receptors.

Inhibiting RNA editing of GluR2 enhances cell death through excitotoxicity

Since under-editing of GluR2 leads to enhanced calcium permeability, it would also be expected to enhance cell death through excitotoxicity. Introducing an R residue into the glutamate receptor of the model genetic organism, *Caenorhabditis elegans*, at a position equivalent to the Q/R site in vertebrate GluRs, results in strong phenotypic impairments including neuronal degeneration.[27] Similarly, mice engineered to be incapable of editing at the Q/R site die shortly after birth from status epilepticus,[28] even though removal of the GluR2 gene is not lethal.[29] Furthermore, preventing all editing by means of siRNA silencing of ADAR2 targeted to the hippocampus in rats leads to degeneration of ischemia-sensitive neurones, which can be rescued by exogenously expressing ADAR2b.[30] These findings support the hypothesis that the presence of AMPARs containing edited GluR2 protect neurones from excitotoxic cell death and that the vulnerability of neurones is attributable to calcium influx through calcium permeable iGluRs.

Is the selective vulnerability of neurones to cell death in ALS caused by excessive calcium influx through AMPARs lacking GluR2 subunits? Although the presence of GluR2 expression in spinal motor neurones has been reported,[31] expression of AMPARs in human or rat spinal motor neurones is low or undetectable.[32–34] Single-cell PCR approaches have shown that, of several neuronal subtypes examined, motor neurones contained the lowest amounts of GluR2 and expression of GluR2 was not significantly altered in ALS,[35] even though AMPA current density in these cells is high, at least in rat spinal motoneurones.[36] However, some studies have shown that both GluR2-containing and non-containing AMPARs exist in

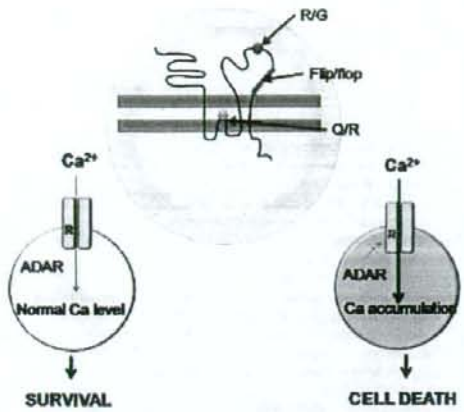

Figure 2. Editing at the Q/R site in the second transmembrane domain of GluR2 ionotropic glutamate receptors by ADAR ("deaminases acting on RNA") reduces the permeability of the channel to calcium ions. Where editing fails, the resultant increase in calcium permeability is thought to contribute to excessive calcium influx when the receptors are activated in the course of synaptic transmission. This can lead in turn to a build-up of excess free calcium in the cytosol, which eventually triggers cell death.

the same cells[37,38] in different membrane synaptic microdomains.[39] This presence of largely calcium-impermeable AMPARs and a subset of calcium-permeable AMPARs results in motor neurones with an overall calcium permeability in response to glutamate intermediate between calcium-permeable and calcium-impermeable AMPARs.[39] In view of the consensus that AMPARs are not highly expressed in spinal motor neurones, it has been suggested[35] that the low levels of GluR2 in motor neurones would provide a phenotype in which changes in calcium permeability due to altered editing would have a greater effect, since even a modest increase in unedited GluR2 subunits would affect a higher proportion of receptors. Because GluR2 knockout mice did not display any neuronal death,[29] an increase of GluR2-lacking AMPA receptors per se cannot induce neuronal death and may merely be an exacerbating factor of excitotoxic neuronal death. This notion is supported by the upregulation of GluR3 (and therefore downregulation of GluR2-containing AMPA receptors) in degenerating motor neurones after long-term intrathecal infusion of kainite in rat[40] and in the spinal cord of *SOD1* transgenic mice,[41,42] which was rescued by GluR2 overexpression.[43] On the other hand, GluR2 under-editing per se induces excitotoxic neuronal death. This difference between GluR2-lacking and unedited GluR2-containing AMPA receptors in their role in excitotoxicity may be attributable to the difference in the functional calcium-permeable AMPA receptor density due to the different efficiency of unedited and edited GluR2 containing AMPA receptor trafficking.[44,45]

The effects of low expression levels of GluR2 on the vulnerability of motor neurones to cell death may also be aggravated by the low levels of calcium-binding proteins in these cells.[46] In goldfish, expression of calcium-binding proteins correlates positively with the expression of calcium-permeable glutamate receptors,[47] and calcium-binding proteins such as calbindin and parvalbumin are absent in motor neurones lost early in ALS but high in less vulnerable motor neurones.[48] In *SOD1*-mice, levels of parvalbumin and calbindin in spinal motor neurones were severely reduced and, in the case of parvalbumin, this preceded symptoms.[49] Taken together, these observations raise the possibility that the vulnerability of motor neurones to cell death in ALS is due to a combination of enhanced calcium entry and reduced buffering capacity of the cells.

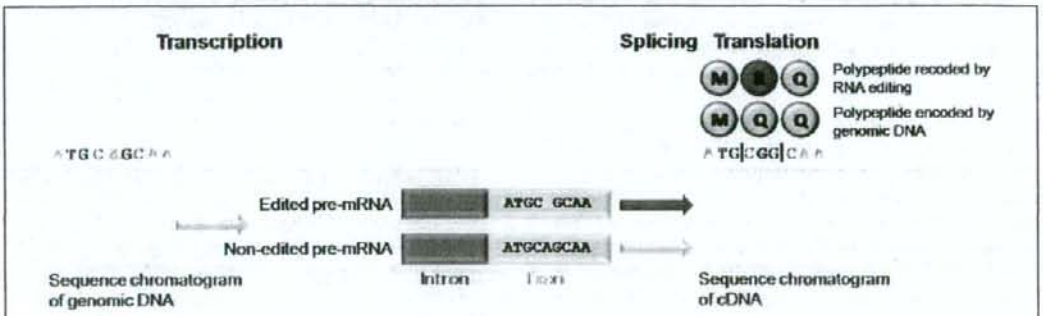

Figure 3. RNA A-to-I editing recodes the genome. Select adenosine (**A**) residues in pre-mRNA are modified to inosine (**I**) by adenosine deaminases acting on RNA (ADARs). Since inosine is interpreted by cellular machineries as guanosine (**G**), A-to-I editing generates transcripts with a nucleotide composition that differs from the corresponding genomic DNA. This has the potential to alter amino acid residues thus generating multiple protein isoforms.

Table 2. Editing sites in iontropic glutamate receptors of the AMPA type, indicating which ADAR enzyme effects editing, where known.

subunit	Edited sites	Editing enzyme	references
GluR 1	None		
GluR 2		ADAR2	67–70
		ADAR2 ADAR1	69–71
GluR 3		Not known	
GluR4		Not known	
GluR5		Not known	
GluR6		ADAR2 ADAR1	72
		Not known	
		Not known	
GluR7	none		

Editing occurs in regions of functional significance: the M1 and M2 transmembrane regions contain residues that control pore conductance and channel properties, and the flip/flop alternatively spliced domain controls the kinetic and pharmacological properties of the receptor.

Unedited AMPA receptors are associated with ALS and neuronal cell death

In 2004, Kwak and colleagues used laser microdissection to isolate single motor neurones and prepared RNA from individuals with ALS and control subjects. Editing efficiency was determined by measuring the difference in digestion patterns of nested GluR2 PCR products with reverse transcription using *BbvI*, which cuts only the unedited RNA.[50,51] Editing of GluR2 receptors in motor neurones was variably reduced ranging from 0% to 100% in sporadic ALS patients, whereas 100% of GluR2 subunits from control subjects were edited.[52] The editing defect was not observed in motor neurones from patients suffering from spinal and bulbar muscular atrophy (SBMA) or from symptomatic mutated *SOD1* transgenic rats[53] or in cerebellar Purkinje cells from patients with dentatorubral–pallido–luysian atrophy (DRPLA)[52] or multiple system atrophy (MSA),[54] neither was it seen in cerebellar Purkinje cells and in motor cortical neurones from sporadic ALS patients, suggesting that the editing defect was specific to motor neurones of sporadic ALS patients. These findings lend support to the view that sporadic ALS involves, at least in part, a motorneurone-specific failure to edit GluR2 subunits, with a resultant excessive calcium entry leading to excitotoxic cell death. This has been lent further support from the observation that transgenic mice engineered to express a GluR2 subunit with an N residue at the Q/R site develop a late-onset ALS-like phenotype.[55]

GluR2: a gatekeeper to neuronal survival

GluR2, through its expression and/or its editing state, appears to act as a "gatekeeper" which can switch the phenotype of cells between two states distinguished by their vulnerability to excitotoxicity. What would be the adaptive advantage of a mechanism in which an error can lead to massive motor

neurone loss? In principle, editing allows a rapid switch in receptor function that can play a role in plasticity or development. Indeed, editing is itself developmentally regulated, with editing of the GluR2 Q/R site beginning in the embryonic stage and continuing throughout life. This is indicated by several observations that death resulting from experimentally imposed editing inefficiency occurs in foetal or early life stages.[28,56] GluR2 Q/R is fully edited in the cerebellum and cerebral cortices of human foetuses and GluR5 increases in editing efficiency from foetus to adult.[51] Kainate receptors on rat DRG (dorsal root ganglion) nociceptor neurones lose their calcium permeability in the first postnatal week, and this coincides with changes in GluR5 editing.[57] In the developing chick embryo, a reduction in calcium permeability over days E6 and E11 is accompanied by an increase in GluR2 expression.[58] The Drosophila ADAR is also highly developmentally regulated.[59] Thus, changes in calcium permeability resulting from editing may match changes in the functional roles of receptors.

Why should it be so important for motor neurones to possess this inbuilt vulnerability that is then mostly held at bay throughout their life by RNA editing? One possibility is that switching off this protection may be important in triggering the fast motor neurone cell death that takes place early in development as overproduced developing motor neurones compete for targets and those that fail are eliminated. This is not likely to be the only explanation for the existence of this mechanism, however, as a calcium-permeable component of ionotropic glutamate responses is present in healthy neurones.[39] It is therefore probable that editing permits a fine-tuning of the calcium influx mediated by glutamate receptors to achieve an end whose benefits balance the risk of excessive calcium influx. For instance, for the electrical phenotype of a neurone to be controlled it may be desirable for the level of neuronal activity to be monitored, and this could be accomplished by an influx of calcium through activated ion channels. Indeed, this has been observed for NMDARs where the level of receptor expression, rather than calcium permeability of individual receptors, is controlled.[60] The GluR2 subunit governs more than just calcium permeability and may influence other mechanisms. For instance, the C-terminal cytoplasmic tail, which is comparatively short in GluR2, mediates subunit interactions as well as interactions with other cytoplasmic proteins which may affect pharmacological properties or the trafficking of the receptor to a specific subcellular location.[61,62]

Is GluR2 a glial gatekeeper too?

In addition to neurones, AMPA receptors are also present in glia and maybe other cells. Although the major contribution of astrocytes to the pathophysiology of ALS appears to derive from dysfunction of glutamate transporters leading to enhanced extracellular glutamate levels,[63] damage to glial cells may combine with dysfunction of neurones through several non-autonomous cell death mechanisms[64] including regulating the expression of GluR2 to control neuronal vulnerability to excitotoxicity.[65] These authors detected reduced glutamate transporter activity in synaptosomes prepared from brain from patients with sporadic ALS. The problem was shown to be attributable to selective loss of the glutamate transporter of astroglia, EAAT2. Knockout of EAAT2 in mice leads to enhanced neuronal activity followed by neuronal death. When compounded with under-editing of GluR2, this will no doubt lead to a synergistic acceleration of excitotoxic neuronal demise. Kwak and colleagues have shown that there is a correlation between the extent of editing at various A-to-I sites and expression levels of ADARs in normal human brains, and found that GluR2 Q/R site-editing was lower in white matter in contrast to the complete editing in gray matter. This indicates that oligodendrocytes express significant amount of Q/R site-unedited GluR2 mRNA, while neurones express solely Q/R site-edited GluR2 mRNA.[66] The presence of calcium-permeable AMPA receptors was also demonstrated in astrocytes.[67] It seems likely that glial cells, in contrast to the majority of neurones, need calcium-permeable AMPA receptors, and GluR2-lacking AMPA receptors are expressed abundantly in astrocytes whereas unedited GluR2-containing AMPA receptors are expressed in oligodendrocytes. Interestingly, a recent report and an earlier study indicate that human malignant glioma cells express under-edited GluR2[68,69] and provide evidence that ADAR1–ADAR2 heterodimer formation may be a regulatory factor determining ADAR2 activity at the GluR2 Q/R site.

Therapeutic prospects for neuroprotection by sustaining RNA editing and 'bolting' the calcium gate

Pre-mRNA A-I editing is emerging as a major determinant of neuronal survival. Reduced levels or under-editing of GluR2 renders cells vulnerable to cell death by excitotoxic calcium influx. To compound this, calcium homeostasis in motor neurones is destabilised by their low calcium-buffering capacity.[49] These developments in understanding RNA editing and its role in ALS may offer prospects for new routes to therapy for ALS based on rescuing the lethality caused by GluR2 under-editing. One such approach might include drug-induced up-regulation of ADAR2 in motor neurones, although it remains to be shown whether overexpression of ADAR2 has its own adverse consequences. More finely targeted approaches might be possible if the factors affecting editing could be determined. One approach to achieving this goal may be the use of suppressor/enhancer screens to identify candidate genes affecting editing. Drosophila in which the single ADAR gene has been knocked out show marked neurodegeneration with accompanying retinal degeneration. Thus, screening EMS mutants or transposon insertions in

a dADAR^{-} background for enhanced or reduced retinal degeneration might identify new genes that regulate editing or compensate for its absence.

The Q/R site is not the only point of A-I editing in GluR2. Editing also occurs at the R/G site to alter desensitization of AMPA receptors without playing a role in excitotoxicity.[70] How might differential editing on the same subunit be achieved? Because some minimal R/G site-editing remains in both heterozygous ADAR1 KO mice[71] and homozygous ADAR2 KO mice,[72] editing at this site may be mediated by both ADAR1 and ADAR2. Alternatively, ADAR2 activity may differ between different classes of neurons, but with different threshold levels for Q/R site editing and R/G site editing. Thus, although ADAR2 activity may vary among motor neurons, it may be kept above the threshold for complete Q/R site-editing, the crucial requirement for survival. Assessment of ADAR2 activity *in vivo* has been hampered by the lack of good markers for ADAR2 activity and differences in the regulatory mechanisms in different cell types. However, the recent discovery that A-I conversion in cytoplasmic FMRP interacting protein (CYFIP2) mRNA is predominantly mediated by ADAR2, and that CYFIP2 mRNA is abundantly expressed in CNS[73] may be useful for assessing ADAR2 activity in neurons.

The discovery of a gatekeeper role for GluR editing motor neuron survival and the link between under-editing and sporadic ALS opens exciting new avenues for new research into the disease. In particular, an improved understanding of the mechanisms controlling editing and the development of improved cell line and animal models may open the way to the development of new therapies for the treatment of this devastating disease.

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Point of View

Newly identified ADAR-mediated A-to-I editing positions as a tool for ALS research

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Abbreviations: 5HT, 5-hytroxytryptamine (serotonine); ADAR, adenosine deaminase acting on RNA; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BLCAP, bladder cancer associated protein; CYFIP2, cytoplasmic fragile X mental retardation protein interacting protein 2; DRPLA, dentatorubro-pallidoluysian atrophy; FLNA, filamin A; hnRNP, heterogeneous nuclear ribonucleoprotein; IGFBP7, insulin-like growth factor binding protein 7; IP, immunoprecipitation; MND, motor neuron disease; PBP, progressive bulbar palsy; RNAi, RNA interference; SBMA, spinal and bulbar muscular atrophy; SCD, spinocerebellar degeneration; SOD1, Cu/Zn superoxide dismutase; TDP-43, transactivation response region DNA-biding protein 43

Key words: RNA editing, ADAR, GluR2, ALS, cell death

Among the extensively occurring adenosine to inosine (A-to-I) conversions in RNA, RNA editing at the GluR2 Q/R site is crucial for the survival of mammalian organisms. Editing at this site is incomplete in the motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS). Adenosine deaminase acting on RNA type 2 (ADAR2) specifically mediates GluR2 Q/R site-editing, hence, it is likely a molecule relevant to the pathogenesis of sporadic ALS. Since no other transcript with ADAR2-mediated A-to-I positions is abundantly expressed in most neurons, the editors at the newly identified A-to-I positions were investigated. CYFIP2 and FLNA mRNAs were identified together with mRNAs having known ADAR2-mediated editing positions in ADAR2-immunoprecipitates of the human cerebellum, indicating that these mRNAs probably possessed ADAR2-mediated positions. Furthermore, an in vitro RNAi knockdown system demonstrated that the CYFIP2 mRNA K/E site and the BLCAP mRNA Y/C site were edited predominantly by ADAR2 and ADAR1, respectively. CYFIP2 mRNA was ubiquitously expressed and particularly abundant in the central nervous system. The extent of CYFIP2 K/E site-editing was between 30% and 80% in the central nervous system. Therefore, the extent of CYFIP2 K/E site-editing may be an additional marker for ADAR2 activity in neuronal and other types of cells in vivo, as well as in vitro, and thus is considered to be a good tool for sporadic ALS research.

A-to-I RNA editing alters the stability, transport or processing of RNA, thereby enhancing the diversity of rather limited genetic information in a region- and even cell type-specific manner. A-to-I conversion occurs most extensively in vertebrate brains and the vast majority occurs in non-coding RNA regions, particularly in the inversely oriented repetitive elements including Alu sequences.[1-3] The important roles of non-coding RNA editing was recently demonstrated in an miRNA system with the alteration of miRNA processing by Drosha-DGCR8 and the generation of new miRNA targeting mRNAs that were different from those targeted by unedited miRNA.[2,4] A-to-I conversion in the coding region of RNA may alter the properties of transmitter-gated ion channels by substituting one amino acid to another as seen in Q/R site-editing of the glutamate receptor subunit.[5] In vertebrates, three structurally related ADARs (ADAR1, ADAR2 and ADAR3) have been identified as enzymes catalyzing the A-to-I conversion. ADAR1 mRNA is widely expressed in various organs where both larger (150-kDa) and smaller (110-kDa) ADAR1 proteins are produced by alternative splicing. ADAR1 is essential for normal development and ADAR1-null mice die in the early embryonic stages.[6] ADAR2 mRNA is widely expressed, most abundantly in the nervous system (Affymetrix HG-U133A:203865_s_at)[7-10] localized in the nucleus. One ADAR2 protein isoform was detected in the mouse brain,[11] whereas alternative splicing of the Alu sequence-containing exon generates two isoforms, ADAR2a and ADAR2b, with a greater abundance in the latter in the human cerebellum.[12] There are limited numbers of A-to-I positions specifically edited by either ADAR1 or ADAR2 in the coding RNA. An investigation on the brains of heterozygous ADAR1-null mice and homozygous ADAR2-null mice indicated that ADAR1 specifically mediates A-to-I conversion of the $5HT_{2c}$ receptor A site,[13,14] while ADAR2 specifically mediates that of the GluR2 Q/R site and the $5HT_{2c}$ receptor C and D sites.[15-25] Recent investigations on the brains of knockout mice and cultured cells using the RNAi system added a new ADAR1-selective A-to-I position in BLCAP mRNA, and the ADAR2-selective positions in mRNAs of CYFIP2 and FLNA.[10,26] ADAR3, a structurally related isoform of ADAR1 and ADAR2, is specifically expressed in the brain but no editing

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activity has been demonstrated in either naturally occurring or artificial substrates.[27,28]

Diseases Associated with Anomalous RNA Editing

A-to-I conversion occurs most extensively in the central nervous system, thereby regulating the expression and properties of receptor/ion channels and the activities of neuronal circuits. Therefore, anomalous RNA editing may result in an abnormal phenotype leading to animal or human diseases affecting the central nervous system.

RNA editing at five A-to-I positions in the 5-hydroxytryptamine 2c (5-HT_{2c}) receptor changes the G-protein-coupled signal transduction in the downstream of the receptor activation and an increase of the extent of editing at the A or the E site has been demonstrated in patients with major depression and in a rat model of depression.[16,17,19,29,30] The editing of A-to-I positions in the 5-HT_{2c} receptor was observed to increase in the victims of suicide among patients with depression or schizophrenia, thus suggesting that 5-HT_{2c} receptor mRNA editing may be associated with changes in mood but not with comorbid psychiatric illnesses.[31] Indeed, the extent of RNA editing at these sites differs among mouse strains and was altered after the administration of antidepressants or exposure to a stressful environment in normal mouse brains.[16,32,33]

An A-to-I conversion of glutamate receptor subunits markedly alters the channel properties of glutamate receptors and hence, the neuronal excitability as a whole. In particular, mutant mice deficient in Q/R site-RNA editing of the AMPA receptor GluR2 subunit exhibit refractory epilepsy and those deficient in Q/R site-editing of the kainate receptor GluR6 subunits become susceptible to epilepsy, as a consequence of an increase of neuronal excitability due to increased Ca^{2+} permeability of these receptors.[34,35] However, no consistent results have been demonstrated as to alteration in editing at these sites in the brains of patients with refractory temporal lobe epilepsy.[36,37]

Several mutations have been identified in the ADAR1 gene in association with family members affected with dyschromatosis symmetrica hereditaria, a dermatologic disease with autosomally dominant transmission.[38] However, whether this skin-affecting disease is induced by a loss of ADAR1 editing function or by a gain of function of the mutated ADAR1 gene has not been demonstrated. Indeed, homozygous ADAR1-null mice die at early embryonic stage and heterozygous ADAR1-null mice are phenotypically normal.[6]

In contrast, motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS) express Q/R site-unedited GluR2 mRNA in variable proportions in a disease-specific and motor neuron-selective manner.[39]

ALS is the most common adult-onset motor neuron disease, characterized by progressive weakness and muscle wasting leading to death within a few years after onset due to the degeneration of both the upper and lower motor neurons. ALS affects healthy subjects abruptly in their mid-life with an incidence of around 1–3 in 100,000 every year. The majority of ALS cases are sporadic, with a variety of phenotypes including limb-onset classical ALS, progressive bulbar palsy (PBP) and ALS with dementia (ALS-D or FTD-MND). About 5–10% of ALS cases are familial, including Cu/Zn superoxide dismutase gene (SOD1)-associated familial ALS (ALS1) that accounts for about 20%, but none of the currently identified gene mutations has been demonstrated to be involved in sporadic ALS. The motor neurons of sporadic ALS patients express various proportions (ranging from 0% to 100%) of GluR2 mRNA lacking A-to-I conversion at the Q/R site.[39] Because inosin in mRNA is read as guanosine during translation, an A-to-I conversion in the Q/R site of GluR2 results in conversion of glutamine (Q:CAG) to arginine (R:CGG), thereby reducing the Ca^{2+} permeability of AMPA receptors containing GluR2 in their tetrameric subunits.[5] The majority of neurons express only Q/R site-edited GluR2 under normal conditions and if A-to-I conversions at this site are incomplete or abolished in artificial conditions, neurons became easily excitable due to an increase in Ca^{2+} influx through AMPA receptors and animals exhibited fatal status epilepticus[34] (Fig. 1). Furthermore, mice transgenic for GluR-B(N), an artificial gene encoding GluR2 with asparagine (N) at the Q/R site, developed motor deficit with a loss of motor neurons after 12 months of age.[40] Because GluR2 with N at the Q/R site works as Q/R site-unedited GluR2 in terms of Ca^{2+} permeability,[41] the results indicate that a substantial increase of Ca^{2+} permeability of AMPA receptors may induce slow progressive death at least in motor neurons (Fig. 1).

Although AMPA receptor-mediated neurotoxicity may play a role in ALS1 as well,[40,42,43] the underlying mechanism is not an increase of Q/R site-unedited GluR2-containing Ca^{2+} permeable AMPA receptors[44] (Table 1) unlike in sporadic ALS, but is likely due to an

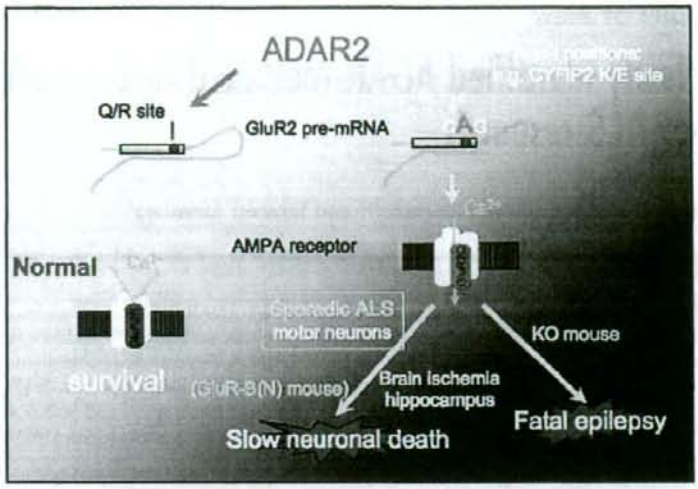

Figure 1. ADAR2 and GluR2 Q/R site-editing. AMPA receptors consist of tetrameric assembly of four subunits and their majority is impermeable to Ca^{2+} because Q/R site-edited GluR2 is included in its assembly. ADAR2 specifically edit the Q/R site of GluR2 pre-mRNA, and a reduction of its activity upregulate Ca^{2+} permeable AMPA receptors with Q/R site-unedited GluR2, which is toxic to neurons. ADAR2 knockout mice exhibit fatal epilepsy, but focal deficiency of ADAR2 activity induces slow neuronal death as seen in motor neurons of sporadic ALS patients and hippocampal pyramidal cells in rats after focal brain ischemia.

Table 1 **GluR2 Q/R site-editing in diseases**

GluR2 mRNA Q/R site	Cortex (%)	motor neurons (%)	cbl/Purkinje cell (%)	hippocampus/pyramidal cells (%)	WM/glial cells (%)
normal human brain[20-25,39]	95–100	100	98–100	100	65–99
sporadic ALS[25,39]	95–100	0–100	98–100	ND	ND
SBMA[44]	ND	100	ND	ND	ND
SCD (DRPLA/MSA)[20,39]	ND	ND	98–100	ND	ND
malignant glioma[61,62]	ND	ND	ND	ND	69–88
normal rat[44,52]	100	100	100	100, 97	100
mSOD1-transgenic rat(G93A, H46R)[44]	ND	ND	100	ND	ND
rat transient forebrain ischemia[52]	ND	ND	ND	7–98	ND

increase of GluR2-lacking Ca^{2+} permeable AMPA receptors. Because GluR2 knockout mice did not display any neuronal death,[45] an increase of GluR2-lacking AMPA receptors per se cannot induce neuronal death and may be an exacerbating factor of excitotoxic neuronal death.[46] Neurotoxicity in mice deficient in GluR2 Q/R site-editing is likely due to an increased density of functional Ca^{2+} permeable AMPA receptors on the synaptic surface resulting from the facilitation of Q/R site-unedited GluR2-containing receptor trafficking.[47,48]

RNA editing at the GluR2 Q/R site is specifically catalyzed by ADAR2 in vertebrates.[15] ADAR2-null mice exhibit fatal status epilepticus[15] as do the mutant mice deficient in GluR2 Q/R site-editing,[34] but these mice display normal behavior when Q/R site-edited GluR2 without ADAR2 activity is expressed by crossing with mutant mice carrying the genetically engineered GluR2 gene encoding arginine (R) instead of glutamine (Q).[15] Therefore, the epileptogenic role of deficient ADAR2 seems to be solely due to deficient editing at the GluR2 Q/R site among various A-to-I positions in both coding[15,49-51] and non-coding RNAs.[4] A reduction of ADAR2 activity in a subset of neurons induces slow progressive neuronal death as demonstrated in the delayed neuronal death of rat hippocampal pyramidal cells after transient ischemia[52] and in the slow progressive death of motor neurons in a conditional ADAR2 knockout mouse.[53] Therefore, the reduction in GluR2 Q/R site-editing in motor neurons of sporadic ALS is likely due to ADAR2 underactivity.[20] Indeed, the expression level of ADAR2 mRNA relative to GluR2 mRNA, a determinant of ADAR2 activity in human white matter,[18] is markedly reduced in the spinal ventral gray matter of sporadic ALS patients,[20,54] thus indicating a reduction of the ADAR2 activity in motor neurons. To demonstrate ADAR2 underactivity in motor neurons of sporadic ALS, a reduction in more than one A-to-I positions that are specifically mediated by ADAR2 may be necessary. However, other than the GluR2 Q/R site, no ADAR2-specific A-to-I position has yet been identified in mRNAs expressed abundantly in the motor neurons.

Novel A-to-I Positions and their Editors

Recently, computational genomic approaches and bioinformatics screening have demonstrated novel A-to-I conversions in four different mRNAs; cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2), filamin A (FLNA), bladder cancer associated protein (BLCAP) and insulin-like growth factor binding protein 7 (IGFBP7).[55] These mRNAs were investigated for specifically ADAR2- or ADAR1-mediated positions because determination of editors at novel A-to-I positions would be useful for analyzing ADARs activities in vivo. An immunoprecipitation (IP) method and an in vitro RNAi knockdown system of ADAR1 and ADAR2 demonstrated that the K/E site in CYFIP2 mRNA and the Y/C site in BLCAP mRNA are edited predominantly by ADAR2 and ADAR1, respectively, and the Q/R site in FLNA mRNA is possibly edited by ADAR2.[10] In brief, CYFIP2, FLNA, GluR2 and kv1.1 mRNAs but not β-actin, BLCAP or IGFBP7 mRNA were recovered from an ADAR2-immunoprecipitate of the nuclear fraction of human cerebellum. Because GluR2 and kv1.1 mRNAs, but not b-actin mRNA, have ADAR2-mediated editing positions, these results suggest that CYFIP2 and FLNA mRNAs, but not BLCAP or IGFBP7 mRNA, have ADAR2-mediated positions. Indeed, in vitro knockdown experiments indicated that the K/E site in CYFIP2 mRNA and the Y/C site in BLCAP mRNA are catalyzed mainly by ADAR2 and ADAR1, respectively (Table 2). Jantsch's lab also reported consistent results from the analysis of the extent of editing by sequencing of cDNAs derived from ADAR2-null mouse brain and primary neuronal culture of ADAR1-null and ADAR1/ADAR2-null mice[26] (Table 2). In accordance with the prediction, they showed that the extent of FLNA Q/R site-editing in ADAR2 null mouse brains is lower than that in control mice. The consistency between the two reports using different methodology strongly suggests that ADAR2 predominantly mediates CYFIP2 K/E site- and FLNA Q/R site-editing and ADAR1 predominantly mediates BLCAP Y/C site- and IGFBP7 K/R site-editing.

A Tool for Sporadic ALS Research

Although normal human motor neurons express only Q/R site-edited GluR2 mRNA,[39] the relative abundance of ADAR2 mRNA markedly differed among neurons,[18] thus suggesting that GluR2 Q/R site-editing may be preserved even in neurons with a relatively low ADAR2 activity. Because the downregulation of the ADAR2 activity is likely an inducer of neuronal death, markers representing a wide range of ADAR2 activity may be a useful tool for detection of the disease onset and evaluation of the efficacy of therapy by ADAR2 upregulation. CYFIP2 mRNA is ubiquitously expressed and is particularly abundant in the central nervous system including motor neurons in the spinal cord (unpublished observation). The extent of CYFIP2 K/E site-editing are in the range of about 30% to 85% in the human brains and spinal cord.[10] Therefore, the extent of CYFIP2 K/E site-editing may become an additional marker for

Table 2 Novel A-to-I positions

	normal mouse brain[26] (%)	ADAR1$^{-/-}$ mouse primary culture[26] (%)	ADAR2$^{-/-}$ mouse brain[26] (%)	human cerebellum[10] (%)	ADAR1 siRNA[10] (%)	ADAR2 siRNA[10] (%)
CYFIP2 K/E site	90	→	↓11	84	↓	0
BLCAP Y/C site	50	↓	33.5	~30	0	→
FLNA Q/R site	16.5	→	↓13.5	0	ND	ND

ADAR2 activity in neuronal and other types of cells in vivo, as well as in vitro. Furthermore, since BLCAP mRNA is abundantly expressed in human brain tissue, the extent of BLCAP Y/C site-editing may become a marker for ADAR1 activity in vivo.

However, The extent of CYFIP2 K/E site-editing and ADAR2 mRNA expression level is not necessarily correlated among human tissues.[10] ADAR2 activity is influenced by several factors including the subcellular localization of ADAR2 protein,[56-58] inositol phosphate 6 (IP-6)[59] and glucose concentration.[60] ADAR2 activity on the GluR2 Q/R site-editing is reduced in human malignant glioma cells[61,62] in which ADAR1 overexpression might reduce the number of active ADAR2 homodimers by facilitating inactive ADAR1/ADAR2 hetero-dimer formation.[62-64] These results suggest that there may be cell type-specific and substrate-specific mechanisms underlying the regulation of ADAR2 activity. However, why ADAR2 is downregulated in motor neurons of sporadic ALS remains to be elucidated.

Recently, abnormally processed TAR DNA-binding protein 43 (TDP-43), a member of hnRNP playing a regulatory role in pre-mRNA splicing,[65-69] was demonstrated to accumulate in cytoplasmic inclusion bodies of motor neurons of patients with sporadic ALS as well as in the cortical neurons of those with frontotemporal lobar degeneration (FTLD),[70,71] but not in cytoplasmic inclusion bodies of motor neurons of patients with SOD1-associated familial ALS.[72,73] Therefore, it is likely that the death-inducing mechanism underlying sporadic ALS may be different from that underlying SOD1-associated familial ALS. On the other hand, several different missense mutations in the TDP-43 gene are found in patients with SOD1-unassociated familial ALS that is clinically and neuropathologically very similar to sporadic ALS.[74] The finding that these mutations were detected only in a small proportion of sporadic ALS cases[74-78] suggests that, although the mechanism underlying aberrant TDP-43 processing is different from TDP-43 gene mutation, the TDP-43 dysfunction resulting from either aberrant protein processing or gene mutation may induce a common neuronal death-inducing cascade. Due to the critical roles that the aberrant TDP-43 processing and ADAR2 under-activity played in the death of motor neurons, the elucidation of a link between these molecular abnormalities may provide a clue to the pathogenesis of sporadic ALS.

The upregulation of ADAR2 activity with normalization of GluR2 Q/R site-editing may become a strategy for ALS therapy, which includes drugs stimulating ADAR2 activity and ADAR2 gene transfer. In such settings, an analysis of RNA editing at newly demonstrated A-to-I positions in CYFIP2 and FLNA mRNA may become a useful tool for evaluating ADAR2 activity and the efficacy of the therapy in vivo, hence a key for opening the door to a cure that has been elusive for patients during the nearly 150 year-long history of ALS research.

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