Flip and Flop Splice Variants of AMPA Receptor Subunits in the Spinal Cord of Amyotrophic Lateral Sclerosis

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ABSTRACT Excitotoxicity mediated by AMPA receptors has been suggested to be implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS). To investigate the relevance of AMPA receptors to motor neuron degeneration in ALS, we evaluated the expression of mRNAs coding for flip and flop splice variants of AMPA receptor subunits (GluR-A to GluR-D) in the cervical segment of the spinal cord from control individuals and patients with ALS using in situ hybridization histochemistry. Transcript mRNAs coding for flop variants were significantly decreased in the ventral horn of the spinal cord from patients with ALS, whereas the mRNAs for flip variants were preserved. These findings suggest that the relative abundance of flip variants vs. flop variants is increased in spinal motor neurons of ALS patients when compared to that of control individuals. Flip variants promote assemblies of slowly desensitizing AMPA receptors. These results imply that spinal motor neurons of ALS patients possess more slowly desensitizing AMPA receptors than those of control individuals. This expression change of AMPA receptors in ALS may account for vulnerability of motor neurons in this disease. **Synapse 45:245–249, 2002.** © 2002 Wiley-Liss, Inc.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of motor neurons in the spinal cord, brainstem, and motor cortex. Although the pathogenesis of sporadic ALS, which accounts for the majority of ALS patients, is still unknown, there are several lines of circumstantial evidence suggesting that AMPA receptor-mediated excitotoxicity contributes to the motor neuron death in ALS (Rothstein, 1996; Shaw and Ince, 1997). This hypothesis is based on the following observations. First, it is suggested that glutamatergic excitotoxicity is elevated in ALS. Increases of glutamate concentrations in serum or cerebrospinal fluid are observed in ALS (Camu et al., 1993; Plaitakis and Caroscio, 1987; Rothstein et al., 1990; Shaw et al., 1995), glutamate concentrations are decreased in the spinal cord tissue from patients with ALS (Malessa et al., 1991; Plaitakis et al., 1988), cerebrospinal fluid from patients with ALS shows neurotoxicity via AMPA receptors (Couratier et al., 1993), and decreases in glutamate uptake capacity are seen in the spinal cord from ALS patients (Rothstein et al., 1992). Second, spinal motor neurons are more vulnerable to AMPA receptor agonists than other spinal neurons in vivo (Hugon et al., 1989; Ikonomidou et al., 1996) and in vitro (Bar-Peled et al., 1999; Carriedo et al., 1996, 2000; Rothstein et al., 1993; Vandenberghe et al., 2000a,b). Thus, both elevated glutamatergic excitotoxicity in ALS and the selective susceptibility of spinal motor neurons to AMPA receptor activation appear to be implicated in the selective motor neuron death in ALS.

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Possibly, functional changes of AMPA receptors expressed by motor neurons of patients with ALS contribute to the motor neuron degeneration. The functions of AMPA receptors vary with their subunit compositions (Bettler and Mulle, 1995). Furthermore, the diversity of AMPA receptor functions also arises from molecular heterogeneity of the subunits (Bettler and Mulle, 1995). Therefore, it is essential to analyze the expression of AMPA receptor subunits and their variants with significant functional implications in ALS spinal cords to understand the relevance of AMPA receptor-mediated excitotoxicity to motor neuron death in ALS.

AMPA receptors are composed of heteromeric complexes of four protein subunits (GluR-A to GluR-D, also known as GluR1 to GluR4) (Hollmann and Heinemann, 1994; Seeburg, 1993). Substantial interest has focused on the possibility that selective vulnerability of human spinal motor neurons results from predominant expression of highly Ca^{2+}-permeable AMPA receptors, brought about by absence of the GluR-B subunit in human spinal motor neurons (Shaw et al., 1999; Williams et al., 1997). We have previously shown that human spinal motor neurons express mRNAs coding for flip and flop variants of the GluR-B subunit (Tomiyama et al., 1996). More recently, it has been reported that GluR-B-containing and GluR-B-lacking AMPA receptors coexist in spinal motor neurons (Vandenberghe et al., 2001). The Ca^{2+}-permeability of AMPA receptors is determined by the presence of GluR-B (Hollman et al., 1991), the only AMPA receptor subunit which is edited at a single site, causing the change of a glutamine to an arginine in the channel pore and thus omitting Ca^{2+} permeability (Sommer et al., 1991). The relative abundance of the edited GluR-B subunit is the main determinant of Ca^{2+}-permeability through AMPA receptors (Geiger et al., 1995). Recently, reduction of this editing efficiency has been found in the ventral horn of ALS spinal cords (Takuma et al., 1999). Another property of AMPA receptors that can modulate the AMPA receptor-mediated excitotoxicity is their desensitization kinetics (Bettler and Mulle, 1995). AMPA receptor desensitization protects neurons against excitotoxic effects of AMPA receptor activation, as demonstrated by the fact that pharmacological block of desensitization enhances AMPA receptor-mediated excitotoxicity to neurons (Brorson et al., 1995; Carriedo et al., 2000; Zorumski et al., 1990), including spinal motor neurons (Ballerini et al., 1995). AMPA receptor desensitization properties arise from different expression patterns of flip/flop alternative splice variants of AMPA receptor subunits (Lambolez et al., 1996; Mosbacher et al., 1994). Indeed, the difference of AMPA receptor desensitization among neuronal cell types has been shown to be an important determinant of selective neuronal vulnerability (Brorson et al., 1995).

The expression differences of flip and flop variants of AMPA receptor subunits between normal individuals and patients with ALS has not been studied in the spinal cord. The aim of the present work was to test the hypothesis that motor neurons of ALS patients are vulnerable, since spinal motor neurons of ALS patients express patterns of flip/flop variants comprising relatively slowly desensitizing AMPA receptors. Therefore, using in situ hybridization histochemistry we studied the expression of mRNAs coding flip/flop alternative splice variants of AMPA receptor subunits in the spinal cord from normal individuals and patients with ALS.

Tissue blocks of the cervical segment of the human spinal cord (C6/7) were obtained at autopsy from six individuals (three men, three women, age range 41–95 years, mean 61.2, SEM 9.6, postmortem delay range 4–17 h, mean 9.6, SEM 2.7), who had no evidence of neurological and/or psychiatric disease, and six patients who died with ALS (one man, five women, age range 45–72 years, mean 63.2, SEM 4.9, postmortem delay range 3–20 h, mean 11.3, SEM 3.0). The ALS patients were clinically diagnosed as sporadic ALS and the diagnosis was confirmed by neuropathological examination. At autopsy, transverse blocks of the spinal cord were taken, frozen on powdered dry ice, and stored. The age and postmortem delay time were not different between the control and ALS groups. Tissue blocks were provided by Prof. A. Probst (Department of Pathology, University of Basel, Switzerland), Dr. J. Pascual (Department of Physiology and Pharmacology, University of Cantabria, Spain), and the Neurological Tissue Bank (University of Barcelona, Hospital Clinic, Barcelona, Spain). Sections (20 μm) were cut using a microtome cryostat.

In situ hybridization was performed as described previously (Tomiyama et al., 1996). The specificity of the oligonucleotide probes that recognize each flip and flop variant of human AMPA receptor subunits, GluR-A to GluR-D, has been validated (Tomiyama et al., 1996). The hybridized sections were exposed to β-max films (Amersham, Arlington Heights, IL) at −70°C with intensifying screens for 4 weeks. For each labeled oligonucleotide probe we included sections which have been hybridized in the presence of 200-fold of the same unlabeled probe, resulting in the complete abolition of the specific hybridization signal; the remaining signal was considered the background level. In addition, we also dipped sections from two ALS patients and two control subjects into autoradiographic emulsion (Kodak NTB2) to confirm the specificity of the hybridization signals by examining silver grains on cells.

Regional intensities of the hybridization signals were determined by reading optical density in film autoradiograms using an image analysis system (MCID; Imaging Research, St. Catharines, Ontario, Canada). Optical density was measured in five subregions, laminae

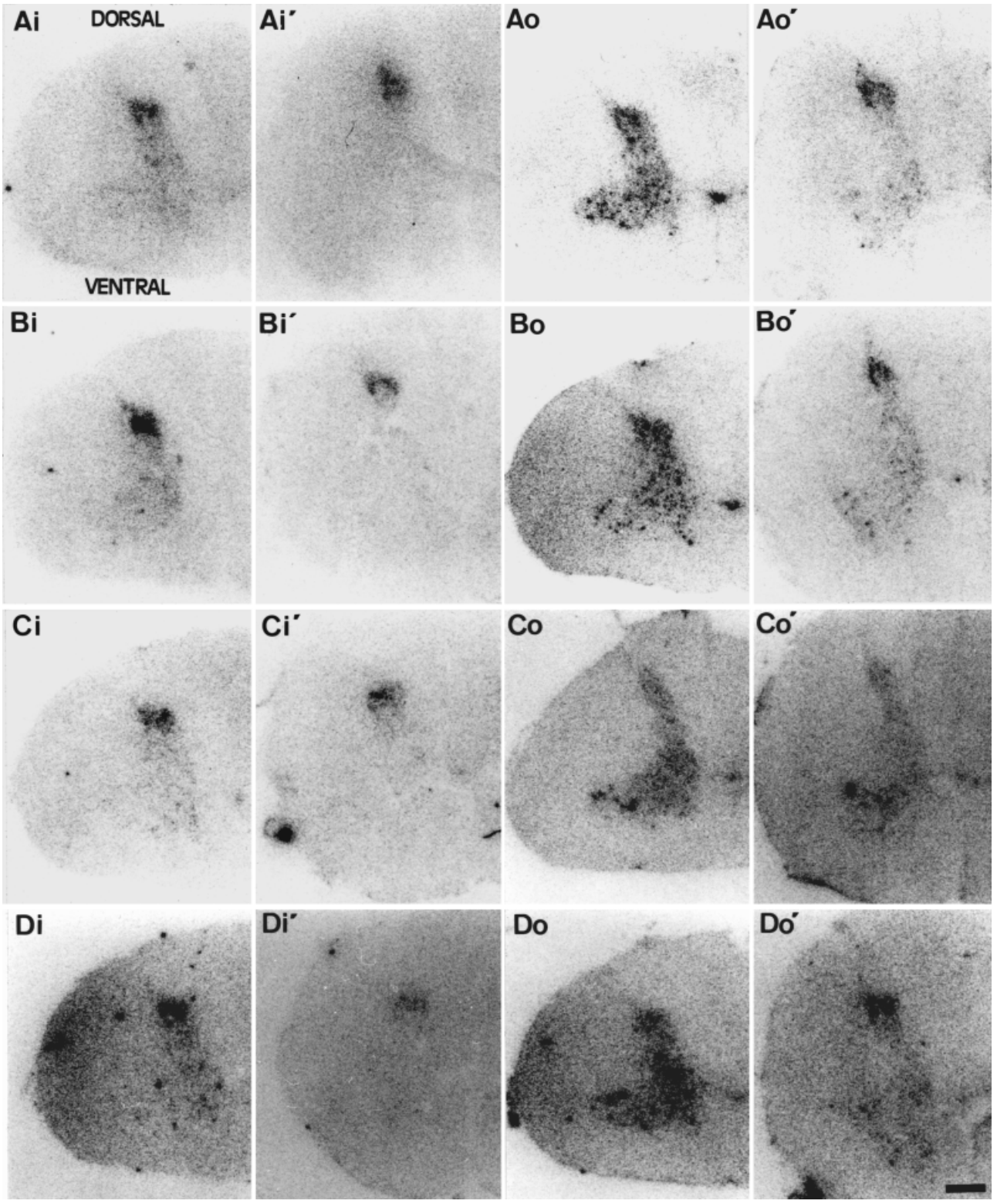

Fig. 1. Distribution of AMPA receptor subunit mRNAs in the cervical segment of the spinal cord from control individuals (Ai-Di and Ao-Do) and patients with amyotrophic lateral sclerosis (Ai'-Di' and Ao'-Do'). Ai, GluR-A Flip mRNA; Ao, GluR-A Flop mRNA; Bi, GluR-B Flip mRNA; Bo, GluR-B Flop mRNA; Ci, GluR-C Flip mRNA; Co, GluR-C Flop mRNA; Di, GluR-D Flip mRNA; Do, GluR-D Flop mRNA. Scale bar = 1 mm.

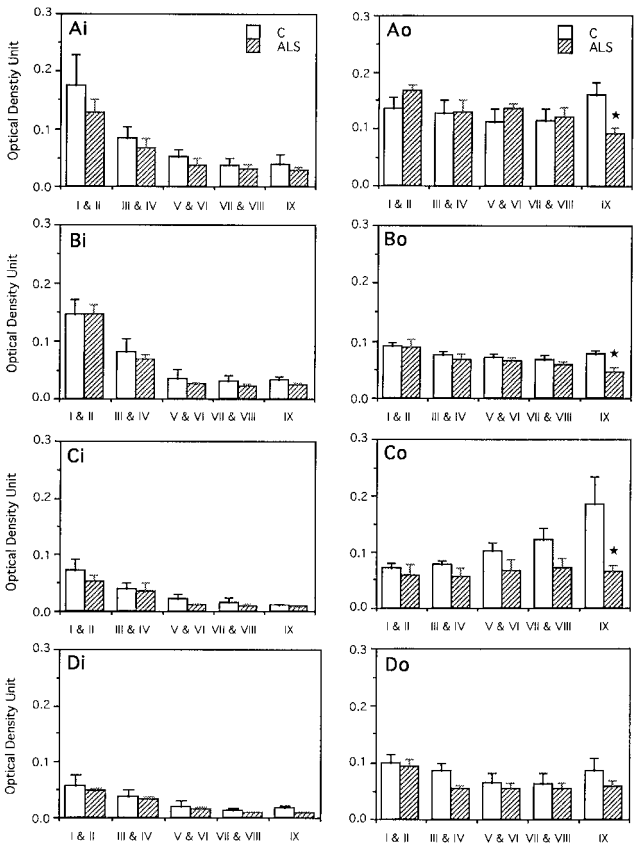

Fig. 2. Relative abundance of AMPA receptor subunit mRNAs in the cervical segment of the spinal cord from control individuals (C) and patients with amyotrophic lateral sclerosis (ALS). Values are given in optical density units (SEM) in the five regions of the spinal cord. Laminae I, II: superficial layers of the dorsal horn; laminae III, IV: deep layers of the dorsal horn; laminae V, VI: dorsal layers of the intermediate gray matter; laminae VII, VIII: ventral layers of the intermediate gray matter; lamina IX: the ventral horn containing the somata of motor neurons. Stars ($P < 0.05$) indicate that the values of ALS were significantly less abundant than those of controls (Mann-Whitney U-test).

I, II; laminae III, IV; laminae V, VI; laminae VII, VIII; and lamina IX. The background density was subtracted from all values. Optical density in each region of the ALS and control groups were compared by the Mann-Whitney U-test. Hybridized sections were stained with cresyl violet for anatomical orientation and motor neuron counting.

The number of large motor neurons per section was 30.6 ± 3.1 (SEM) and 11.2 ± 1.4 (SEM) in the cervical segment of the spinal cord from control individuals and ALS patients, respectively (−63% in ALS, $P < 0.0002$, *t*-test). Quantitative analyses of film autoradiograms revealed that most transcripts were decreased in most of regions in the cervical segment of the ALS group compared to the control group (Fig. 1). The expression of mRNA transcripts coding for flop variants, GluR-A Flop (−43%), GluR-B Flop (−41%), and GluR-C Flop (−66%), was significantly decreased only in lamina IX (the ventral horn) of the ALS group (Fig. 2). Whereas hybridization signal for flip variants was preserved in the ventral horn of the spinal cord from patients with ALS, the number of motor neurons was decreased. Additionally, no significant changes in the expression of AMPA receptor subunit mRNAs were observed in the intermediate gray matter and dorsal horn (Fig. 2). The expression of the mRNAs coding GluR-B flip and GluR B flop subunits was identified by black spots in the ventral horn (Fig. 1Bi, 1Bo).

We have demonstrated that there is a selective reduction of the mRNA coding for flop splice variants of AMPA receptor subunits in the ventral horn of the cervical segment of the spinal cord from patients with ALS. Microscopic examination of the human spinal cord revealed a considerable number of neurons in the ventral horn, indicating that the hybridization signals in the ventral horn could originate from both glial cells and neurons, including motor neurons. Moreover, we and another group have demonstrated that the motor neuron is the main expression site of AMPA receptor mRNAs in human ventral horn (Tomiyama et al., 1996; Williams et al., 1997). In the present study, motor neurons exclusively showed silver grain clustering. On the other hand, glial cells and neurons other than motor neurons in the ventral horn did not have silver grain clustering (data not shown). Accordingly, the hybridization signal level in the ventral horn most probably reflected the expression of the mRNAs on motor neurons.

Additionally, we showed that human motor neurons presumably displayed transcript mRNA for GluR-B subunits. However, selective reduction in the expression of mRNA coding for GluR-B subunits was not seen in the ventral horn of ALS patients, suggesting that a difference of Ca^{2+} permeability of AMPA receptors does not play an important role to determine the vulnerability of motor neurons in ALS patients.

A decrease in AMPA receptor subunit mRNA content in the spinal cord from ALS patients has been reported (Virgo et al., 1996). The authors analyzed the expression of the mRNAs coding for flop variants in homogenates of the entire spinal cord and also showed a decrease in the levels of the mRNA coding for GluR-A and GluR-B in ALS spinal cord. Our observations are in good agreement with those results. In addition, we showed that the decrease of AMPA receptor subunit mRNAs in the spinal cord resulted from the reduction of the mRNA content in the spinal ventral horn.

Our finding suggests that motor neurons of patients with ALS express relatively abundant flip variants of AMPA receptor subunits compared with those of normal individuals. Those AMPA receptors composed of more flip variants display slower desensitization kinetics to glutamate (Lambolez et al., 1996; Mosbacher et al., 1994). It has been shown that slow desensitization of AMPA receptors renders the neuron vulnerable to AMPA receptor agonists (Brorson et al., 1995; Carriedo

et al., 2000; Zorumski et al., 1990). Accordingly, this finding suggests that spinal motor neurons of patients with ALS may have AMPA receptors with slower desensitizing kinetics than those of controls. AMPA receptor desensitization appears not to explain the differential vulnerability of motor neurons and sensory neurons to AMPA receptor agonists in normal rats (Vandenberghe et al., 2000a). However, it remains to be determined why motor neurons in the ALS disease process are progressively degenerating. This expression difference in splice variants of AMPA receptor subunits in the spinal cord of ALS patients may be one of the causes that account for the vulnerability of spinal motor neurons in ALS.

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