Supplementary Data

Materials and Methods

ADAR2 in degenerating neurons in other neurological diseases

Formalin-fixed paraffin-embedded sections at the level of the pontine nuclei were obtained from patients with ALS (case 4 in the Table), multiple system atrophy (73 y, female), or spinocerebellar atrophy type 1 (53 y, male) to examine the disease specificity of the alteration in ADAR2 expression in ALS spinal motor neurons. Immunohistochemistry was performed as described in the Materials and Methods section in the text.

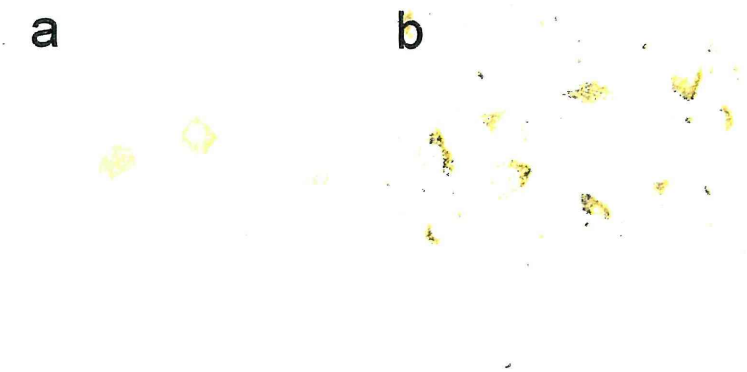
Immunohistochemistry with several different anti-ADAR2 antibodies.

In addition to the RED1 antibody, another anti-ADAR2 antibodies, C-15 (Santa Cruz Biotechnology, Santa Cruz, CA), was used. The antibody was diluted (1:100) and incubated with the samples overnight at 4°C. The C-15 ADAR2 antibody recognizes the long C-terminus. This experiment was done using the spinal cord sections from a control subject (case 12 in the Table).

Supplementary Figure 1

ADAR2 immunostaining in degenerating neurons in other neurological diseases. Neurons in the pontine nuclei of an ALS patient exhibit slight ADAR2 immunoreactivity in the cytoplasm (a). The neurons in the pontine nuclei in both multiple system atrophy (b) and spinocerebellar atrophy type 1 (c) showed faint ADAR2 immunoreactivity, although these neurons were atrophic and reduced in number. These results suggested that the alteration of ADAR2 activity was not involved in the process of neuronal death in the pontine nucleus of MSA or SCA1. Bar indicates 20 μm.

Supplementary Figure 2



Immunohistochemistry with two anti-ADAR2 antibodies. Both RED1 (a) and C-15 (b) stained specifically the cytoplasm but not the nucleus of motor neurons. Non-specific lipofuscin staining is observed in (b).

J Pharmacol Sci

Journal of Pharmacological Sciences

Forum Minireview

Novel Etiological and Therapeutic Strategies for Neurodiseases: RNA Editing Enzyme Abnormality in Sporadic Amyotrophic Lateral Sclerosis

Takuto Hideyama[1,*a], Takenari Yamashita[1], Yoshinori Nishimoto[2], Takeshi Suzuki[3], and Shin Kwak[1,*b]

[1]*Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[2]*Deptartment of Neurology, School of Medicine, Keio University, 35 Shinanomati, Shinjuku-ku, Tokyo 160-8582, Japan*
[3]*Division of Basic Biological Sciences, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*

Received October 20, 2009; Accepted March 5, 2010

Abstract. The motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS) express abundant Q/R site–unedited GluR2 mRNA, whereas those of patients with other motor neuron diseases including familial ALS associated with mutated SOD1 (ALS1) and those of normal subjects express only Q/R site–edited GluR2 mRNA. Because adenosine deaminase acting on RNA type 2 (ADAR2) specifically catalyzes GluR2 Q/R site–editing, it is likely that ADAR2 activity is not sufficient to edit this site completely in motor neurons of patients with sporadic ALS. Because these molecular abnormalities occur in disease- and motor neuron–specific fashion and induce fatal epilepsy in mice, we have hypothesized that GluR2 Q/R site–underediting due to ADAR2 underactivity is a cause of neuronal death in sporadic ALS. We found that cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) mRNA had an ADAR2-mediated editing position using RNA interference knockdown. Our review will include a discussion of new ADAR2 substrates that may be useful for research on sporadic ALS.

Keywords: RNA editing, amyotrophic lateral sclerosis (ALS), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, GluR2 Q/R, cytoplasmic fragile X nuclear retardation protein interacting protein 2 (CYFIP2), neurodisease

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive paralysis with muscle wasting due to selective loss of upper and lower motor neurons. More than 90% of cases of ALS are sporadic, while the remaining cases of ALS have more than one other affected family member (familial ALS). Gene mutations causative of familial ALS, including those of the superoxide dismutase 1 (SOD1) gene, have not been detected in the majority of sporadic cases of ALS (1), indicating that the pathogenesis of sporadic ALS differs from that of familial ALS. Several hypotheses concerning the pathogenesis of sporadic ALS have been suggested, including those related to excitotoxicity (2), toxicity (3), autoimmunity (4), infection (5), and oxidative stress (6). Among these, considerable evidence supports the excitotoxic hypothesis (7 – 9).

The mechanism of initiation of motor neuronal death appears to involve excessive influx of Ca^{2+} through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (10). The determinants of this Ca^{2+} influx include the Ca^{2+} permeability of AMPA receptors, which is due to the presence of the GluR2 subunit and related to the reduction of GluR2 Q/R editing, and the density of receptors on the cell surface, which is regulated by many factors including regulatory proteins, direct

Corresponding authors.
*[a]hideyamat-int@h.u-tokyo.ac.jp, *[b]kwak-tky@umin.net
Published online in J-STAGE
doi: 10.1254/jphs.09R21FM

phosphorylation, and RNA editing at the GluR2 Q/R site (Fig. 1) (11).

We have demonstrated that RNA editing of GluR2, a subunit of the AMPA receptor, at the Q/R site is decreased in motor neurons of a small number of sporadic ALS cases in disease-specific and neuronal class–selective fashion (12, 13). Functional AMPA receptors are tetrameric assemblies of GluR1, GluR2, GluR3, and GluR4, in various combinations produced in nonstochastic fashion. In mammals, all GluR2 mRNAs in neurons are completely edited and the majority of AMPA receptors have GluR2 in their composition, making AMPA receptors Ca^{2+}-impermeable. In the motor neurons in patients with ALS, due to underediting of GluR2 mRNA at the Q/R site, the proportion of Ca^{2+}-permeable AMPA receptors may be increased, resulting in neuronal death (14) . RNA editing at the GluR2 Q/R site is specifically catalyzed by ADAR2 (15). Reduction of this enzyme activity is probably the cause of the underediting observed in ALS motor neurons.

2. RNA editing and ADARs

RNA editing is a posttranscriptional modification of mRNA that alters the amino acids specified by the gene. The resulting change in amino acid residues alters the biological function of translated molecules; this is most clearly demonstrated in alterations of channel properties including those of the Ca^{2+} permeability of GluRs, a subunit of AMPA, and kainate receptors (16, 17). In human and rodent brains, the efficiency of editing at each editing site of GluRs is developmentally and regionally regulated (18 – 24), and abnormal RNA editing may result in animal or human diseases affecting the central nervous system. However, no consistent findings have been reported regarding alteration of these editing sites in the case of temporal lobe epilepsy (25), depression (26), and schizophrenia (27). In contrast, abnormal editing has been demonstrated to be associated with certain neurological diseases including amyotrophic lateral sclerosis (28, 29).

These alterations are catalyzed by the adenosine deaminases, which recognize a specific target sequence of nucleotides and convert an adenosine (A) to an inosine (I), which the ribosome translates as a guanosine (G).

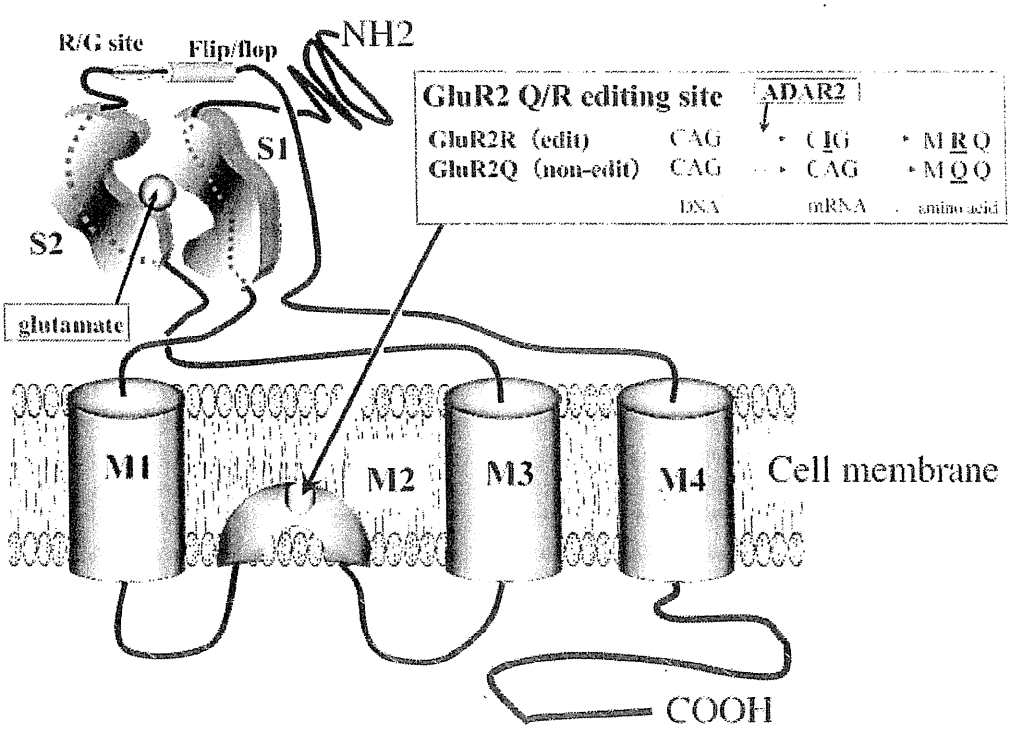


Fig. 1. Structure of the GluR subunit. GluR2 subunit has four membrane domains (M1 – M4). The Q/R site (Q/R) is located in the M2 domain and this editing site is the determinant of Ca^{2+} permeability of the AMPA receptor. The R/G site (R/G) and the flip/flop alternative splicing site are located between the M3 and M4 domains, and these sites are the determinants of channel desensitization.

This A-to-I RNA editing controls a variety of biologically important mRNAs and is specifically catalyzed by either adenosine deaminase acting on RNA type 1 (ADAR1) or type 2 (ADAR2) in mammals including humans. A lot of novel A-to-I editing sites have been identified in vitro in mRNAs abundantly expressed in mammalian organs by means of computational genomic techniques (30), although the enzyme catalyzing editing at these sites has yet to be determined.

ADAR2 predominantly catalyzes RNA editing at the Q/R site of GluR2 both in vivo and in vitro (15, 31, 32), whereas both ADAR1 and ADAR2 catalyze the Q/R sites of GluR5 and GluR6, which are subunits of kainate receptors (15, 32). ADAR3, a third member of the ADAR family, is exclusively expressed in the central nervous system but is catalytically inactive on both extended dsRNA and known pre-mRNA editing substrates (33, 34).

3. New substrates of ADAR2

Using immunoprecipitation and the RNA interference (RNAi) knockdown system in vitro, we investigated whether the recently reported A-to-I editing sites in CYFIP2, filamin A (FLNA), bladder cancer associated protein (BLCAP), and insulin-like growth factor binding protein 7 (IGFBP7) mRNAs (35) are the substrates of ADAR1 or ADAR2 in humans. We also examined whether these mRNAs form complexes with ADAR2 in humans, by means of ADAR2-immunoprecipitation of nuclear extracts of human cerebellum (36).

Using RNAi knockdown, we found that CYFIP2 mRNA had an ADAR2-mediated editing position and that BLCAP mRNA had an ADAR1-mediated editing position (Table 1) (36, 37). In addition, we found that ADAR2 formed complexes with mRNAs with ADAR2-mediated editing positions including GluR2, kv1.1, and CYFIP2 mRNAs, particularly when the editing sites were edited in human cerebellum by means of the immunoprecipitation method. CYFIP2 mRNA was ubiquitously expressed in human tissues with variable extents of K/E site-editing (Fig. 2) (36, 37).

Table 1. Novel A-to-I positions

Editing site	CYFIP2 K/E	BLCAP Y/C	Reference
Normal mouse brain (%)	90	50	(38)
Mouse neuronal primary culture (%)	54.5	36.5	(38)
ADAR1$^{-/-}$ mouse neuronal primary culture (%)	50	8.5	(38)
ADAR2$^{-/-}$ mouse brain (%)	approx. 11	33.5	(38)
Human cerebellum (%)	84	approx. 30	(37)
Hela cell (%)	1.9	13	(37)
ADAR1 siRNA in Hela cell (%)	0.4 – 1.1	0	(37)
ADAR2 siRNA in Hela cell (%)	0	16 – 18	(37)

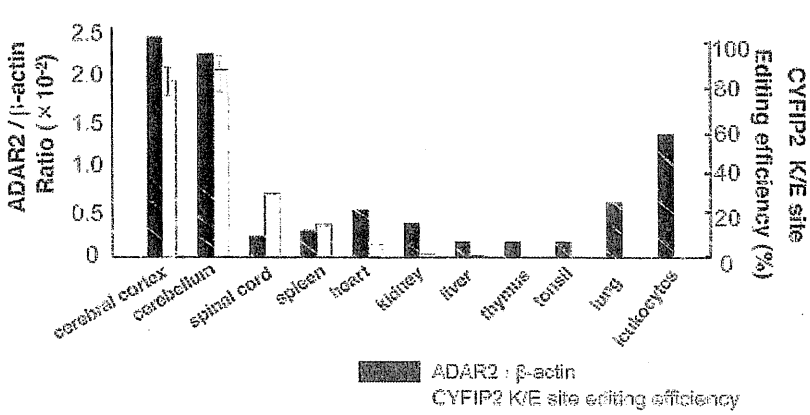


Fig. 2. Extent of CYFIP2-mRNA editing and level of expression of ADAR2 mRNA in human tissues. Tissues with high expression levels of ADAR2 mRNA in the β-actin mRNA base (black columns) tend to show higher extent of RNA editing at the CYFIP2 K/E site (gray columns) than those with low ADAR2 mRNA expression level, whereas some regions with high ADAR2 mRNA expression level (e.g., leukocytes) showed very low extents of CYFIP2 mRNA editing. Value represents the mean ± S.D. for multiple samples of cerebral cortex (n = 4), cerebellum (n = 5), and leukocytes (n = 7) and represents the mean for the rest of tissue samples (n < 3). Reproduced from Ref. 37.

4. Conclusion

CYFIP2 mRNA is particularly abundant in the central nervous system including motor neurons in the spinal cord, and the extent of site-editing in it ranges from 30% to 85% in the human central nervous system (37, 38). Because ADAR2 underactivity may be a cause of death of motor neurons in sporadic ALS, the CYFIP2 K/E site, a newly identified ADAR2-mediated editing position, may become a useful tool for ALS research. To investigate whether deficiency of ADAR2 activity induces slow neuronal death as seen in motor neurons of sporadic ALS patients, we have generated genetically modified mice in which the ADAR2 gene is conditionally knocked out in motor neurons using the Cre-loxP system.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan (H18-Kokoro-017, SK); from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (18023012, SK); a grant from The Nakabayashi Trust for ALS Research (TH); and a grant from the Japan ALS association (TH).

References

1 Jackson M, Al-Chalabi A, Enayat ZE, Chioza B, Leigh PN, Morrison KE. Copper/zinc superoxide dismutase 1 and sporadic amyotrophic lateral sclerosis: analysis of 155 cases and identification of a novel insertion mutation. Ann Neurol. 1997;42:803–807.

2 Rothstein J, Tsai G, Kuncl R, Clawson L, Cornblath D, Drachman D, et al. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol. 1990;28:18–25.

3 Durlach J, Bac P, Durlach V, Durlach A, Bara M, Guiet-Bara A. Are age-related neurodegenerative diseases linked with various types of magnesium depletion? Magnes Res. 1997;10:339–353.

4 Hoffman PM, Robbins DS, Oldstone MB, Gibbs CJ Jr, Gajdusek DC. Humoral immunity in Guamanians with amyotrophic lateral sclerosis and parkinsonism-dementia. Ann Neurol. 1981;10:193–196.

5 Moulignier A, Moulonguet A, Pialoux G, Rozenbaum W. Reversible ALS-like disorder in HIV infection. Neurology. 2001;57:995–1001.

6 Rao AV, Balachandran B. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr Neurosci. 2002;5:291–309.

7 Allaoua H, Chaudieu I, Krieger C, Boksa P, Privat A, Quirion R. Alterations in spinal cord excitatory amino acid receptors in amyotrophic lateral sclerosis patients. Brain Res. 1992;579:169–172.

8 Vandenberghe W, Robberecht W, Brorson J. AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. J Neurosci. 2000;20:123–132.

9 Rothstein JD, Kuncl RW. Neuroprotective strategies in a model of chronic glutamate-mediated motor neuron toxicity. J Neurochem. 1995;65:643–651.

10 Kwak S, Weiss JH. Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. Curr Opin Neurobiol. 2006;16:281–287.

11 Kawahara Y, Kwak S. Excitotoxicity and ALS: what is unique about the AMPA receptors expressed on spinal motor neurons? Amyotroph Lateral Scler Other Motor Neuron Disord. 2005;6:131–144.

12 Takuma H, Kwak S, Yoshizawa T, Kanazawa I. Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. Ann Neurol. 1999;46:806–815.

13 Kawahara Y, Ito K, Sun H, Aizawa H, Kanazawa I, Kwak S. Glutamate receptors: RNA editing and death of motor neurons. Nature. 2004;427:801.

14 Kwak S, Kawahara Y. Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. J Mol Med. 2005;83:110–120.

15 Higuchi M, Maas S, Single F, Hartner J, Rozov A, Burnashev N, et al. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature. 2000;406:78–81.

16 Burnashev N, Monyer H, Seeburg P, Sakmann B. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron. 1992;8:189–198.

17 Köhler M, Burnashev N, Sakmann B, Seeburg P. Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. Neuron. 1993;10:491–500.

18 Paschen W, Djuricic B. Extent of RNA editing of glutamate receptor subunit GluR5 in different brain regions of the rat. Cell Mol Neurobiol. 1994;14:259–270.

19 Paschen W, Schmitt J, Dux E, Djuricic B. Temporal analysis of the upregulation of GluR5 mRNA editing with age: regional evaluation. Brain Res Dev Brain Res. 1995;86:359–363.

20 Paschen W, Djuricic B. Regional differences in the extent of RNA editing of the glutamate receptor subunits GluR2 and GluR6 in rat brain. J Neurosci Methods. 1995;56:21–29.

21 Schmitt J, Dux E. Gissel C, Paschen W. Regional analysis of developmental changes in the extent of GluR6 mRNA editing in rat brain. Brain Res DevelopBrain Res. 1996;91:153–157.

22 Bernard A, Ferhat L, Dessi F, Charton G, Represa A, Ben-Ari Y, et al. Q/R editing of the rat GluR5 and GluR6 kainate receptors in vivo and in vitro: evidence for independent developmental, pathological and cellular regulation. Eur J Neurosci. 1999;11:604–616.

23 Kawahara Y, Ito K, Sun H, Kanazawa I, Kwak S. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. Eur J Neurosci. 2003;18:23–33.

24 Barbon A, Vallini I, La Via L, Marchina E, Barlati S. Glutamate receptor RNA editing: a molecular analysis of GluR2, GluR5 and GluR6 in human brain tissues and in NT2 cells following in vitro neural differentiation. Brain Res Mol Brain Res. 2003;117:168–178.

25 Kortenbruck G, Berger E, Speckmann EJ, Musshoff U. RNA editing at the Q/R site for the glutamate receptor subunits GLUR2, GLUR5, and GLUR6 in hippocampus and temporal cortex from epileptic patients. Neurobiol Dis. 2001;8:459–468.

26 Englander MT, Dulawa SC, Bhansali P, Schmauss C. How stress

and fluoxetine modulate serotonin 2C receptor pre-mRNA editing. J Neurosci 2005;25:648–651.

27 Iwamoto K, Bundo M, Kato T. Estimating RNA editing efficiency of five editing sites in the serotonin 2C receptor by pyrosequencing. Rna 2005;11:1596–1603.

28 Takuma H, Kwak S, Yoshizawa T, Kanazawa I. Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. Ann Neurol. 1999;46:806–815.

29 Kwak S, Takuma H, Kanazawa I. Molecular mechanism and therapeutics of amyotrophic lateral sclerosis. San Diego, CA: Elsevier Science B.V.; 2001.

30 Li JB, Levanon EY, Yoon JK, Aach J, Xie B, Leproust E, et al. Genome-wide identification of human RNA editing sites by parallel DNA capturing and sequencing. Science. 2009;324:1210–1213.

31 Melcher T, Maas S, Herb A, Sprengel R, Seeburg P, Higuchi M. A mammalian RNA editing enzyme. Nature. 1996;379:460–464.

32 Wang Q, Khillan J, Gadue P, Nishikura K. Requirement of the RNA editing deaminase ADAR1 gene for embryonic erythropoiesis. Science. 2000;290:1765–1768.

33 Melcher T, Maas S, Herb A, Sprengel R, Higuchi M, Seeburg PH. RED2, a brain-specific member of the RNA-specific adenosine deaminase family. J Biol Chem. 1996;271:31795–31798.

34 Chen CX, Cho DS, Wang Q, Lai F, Carter KC, Nishikura K. A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains. RNA. 2000;6:755–767.

35 Levanon EY, Hallegger M, Kinar Y, Shemesh R, Djinovic-Carugo K, Rechavi G, et al. Evolutionarily conserved human targets of adenosine to inosine RNA editing. Nucleic Acids Res. 2005;33:1162–1168.

36 Kwak S, Nishimoto Y, Yamashita T. Newly identified ADAR-mediated A-to-I editing positions as a tool for ALS research. RNA Biol. 2008;5:193–197.

37 Nishimoto Y, Yamashita T, Hideyama T, Tsuji S, Suzuki N, Kwak S. Determination of editors at the novel A-to-I editing positions. Neurosci Res. 2008;61:201–206.

38 Riedmann EM, Schopoff S, Hartner JC, Jantsch MF. Specificity of ADAR-mediated RNA editing in newly identified targets. RNA. 2008;14:1110–1118.

NEUROPATHOLOGY

Neuropathology 2010; **30**, 182–188 doi:10.1111/j.1440-1789.2009.01090.x

Symposium: Advances in amyotrophic lateral sclerosis research

AMPA receptor-mediated neuronal death in sporadic ALS

Shin Kwak,[1] Takuto Hideyama,[1] Takenari Yamashita[1] and Hitoshi Aizawa[2]

[1]Department of Neurology, Graduate School of Medicine, The University of Tokyo, Tokyo and [2]Division of Neurology, Department of Internal Medicine, Asahikawa Medical College, Hokkaido, Japan

α - amino - 3 - hydroxy - 5 - methyl - 4 - isoxazolepropionate (AMPA) receptor-mediated excitotoxicity has been proposed to play a role in death of motor neurons in amyotrophic lateral sclerosis (ALS). We demonstrated that RNA editing of GluR2 mRNA at the glutamine/arginine (Q/R) site was decreased in autopsy-obtained spinal motor neurons, but not in cerebellar Purkinje cells, of patients with sporadic ALS. This molecular change occurs in motor neurons of sporadic ALS cases with various phenotypes, but not in degenerating neurons of patients with other neurodegenerative diseases, including SOD1-associated familial ALS. Because GluR2 Q/R site-editing is specifically catalyzed by adenosine deaminase acting on RNA 2 (ADAR2), it is likely that regulatory mechanism of ADAR2 activity does not work well in the motor neurons of sporadic ALS. Indeed, ADAR2 expression level was significantly decreased in the spinal ventral gray matter of sporadic ALS as compared to normal control subjects. It is likely that ADAR2 underactivity selective in motor neurons induced deficient GluR2 Q/R site-editing, which results in the neuronal death of sporadic ALS. Thus, among multiple different molecular mechanisms underlying death of motor neurons, it is likely that an increase of the proportion of Q/R site-unedited GluR2-containing Ca^{2+}-permeable AMPA receptors initiates the death of motor neurons in sporadic ALS. To this end, normalization of ADAR2 activity in motor neurons may become a therapeutic strategy for sporadic ALS.

Key words: ADAR2, ALS, AMPA receptor, GluR2, RNA editing.

Correspondence: Shin Kwak, MD, PhD, Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: kwak-tky@umin.ac.jp

Received 30 October 2009 and accepted 10 November 2009; published online 19 January 2010.

THE AMPA RECEPTOR-MEDIATED NEURONAL DEATH HYPOTHESIS

Molecular mechanisms leading motor neurons to death have not been elucidated in ALS, even in SOD1-associated familial ALS (ALS1) on which a tremendous number of investigations have been conducted for more than a decade. Although different causative genes have been identified in several different familial ALS, the majority of sporadic ALS cases do not carry mutations in these causative genes, indicating that none of them play a causal role in sporadic cases.[1] Therefore, it is likely that there are multiple different death pathways in motor neurons. Among the hypotheses proposed to explain the etiology of sporadic ALS, 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 cultured spinal cord neurons. Although mechanisms underlying excitotoxic neuronal injury are complex and incompletely understood, intracellular Ca^{2+} overload is an important trigger and an increased influx of Ca^{2+} through activated AMPA receptor-coupled channels appears to play a key role in slow death of motor neurons in culture.[2–4]

MOLECULAR MECHANISM UNDERLYING AMPA RECEPTOR-MEDIATED NEUROTOXICITY IN SPORADIC ALS

Functional AMPA receptors are tetrameric assemblies that are composed of four subunits, GluR1, GluR2, GluR3 and GluR4, in various combinations. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether the receptor has the GluR2 subunit in its subunit assembly. AMPA receptors that contain at least one GluR2 subunit

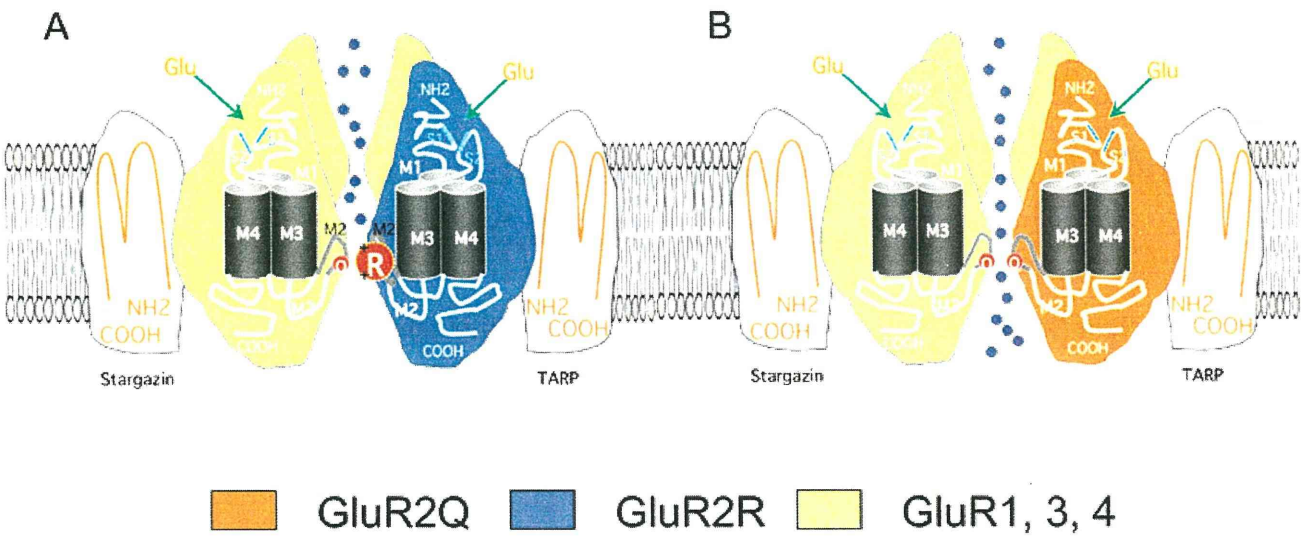


Fig. 1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor and Ca^{2+}-permeability. A. AMPA receptor containing GluR2 subunit edited at the glutamine/arginine (Q/R) site (blue) in M2 (curved line in dark gray). Due to the positive charge of arginin (R) residue at the Q/R site, Ca^{2+} (blue circle) cannot pass through the channel pore. Majority of the AMPA receptors expressed on neurons is Q/R site-edited GluR2-containing AMPA receptors. B. Q/R site-unedited GluR2-containing (orange) or GluR2-lacking AMPA receptors. When none of the subunits have R at the Q/R position, AMPA receptors are Ca^{2+}-permeable. GluR1, 3, 4 subunits are in yellow.

have low Ca^{2+} conductance (Fig. 1A), whereas those lacking a GluR2 subunit are Ca^{2+} permeable.[5] These properties of GluR2 are generated by a single nucleotide conversion from adenosine (A) to inosine (I) by post-transcriptional RNA processing called RNA editing (A-to-I editing), during which inosine is recognized as guanosine during translation, glutamine (Q) codon (CAG) is substituted by arginine (R) codon (CIG; CGG) at the position called the Q/R site in the putative second membrane domain (M2) (Fig. 1B).[6] Analyses of adult rat, mouse and human brain RNA have demonstrated that almost all GluR2 mRNA *in vivo* has R at the Q/R site, whereas Q remains at this critical position in the GluR1, GluR3 and GluR4 subunits. The change in amino acid residue at the Q/R site of GluR2 results in marked alterations in channel properties of AMPA receptors, including Ca^{2+} permeability,[7–9] trafficking,[10] subunit assembly[11] and kinetic aspects of channel gating.[12] Furthermore, failure of GluR2 Q/R site-editing led mice to fatal status epilepticus.[13] Therefore, reductions in both GluR2 expression and GluR2 Q/R site-editing increase Ca^{2+} influx through AMPA receptor-coupled ion channels. We found that extents of GluR2 Q/R site-editing, but not of GluR2 mRNA expression were decreased in motor neurons of sporadic ALS. Because mice deficient for GluR2 Q/R site-editing died young from status epilepticus,[13] an increase of Q/R site-unedited AMPA receptors is likely a death-inducing molecular change in sporadic ALS.

Motor neurons undergo progressive degeneration in ALS, while other neuronal subsets undergo degeneration at a much later disease stage, if ever. We found that the reduction of GluR2 Q/R site-editing did not occur in the cerebellar Purkinje cells of patients with sporadic ALS.[14] Additionally, we found that dying cerebellar Purkinje cells of patients with spinocerebellar degeneration (i.e. dentatorubral pallidoluysian atrophy (DRPLA) and multiple system atrophy (MSA-c)) expressed only normally Q/R site-edited GluR2 mRNA.[14] Moreover, GluR2 Q/R site-editing has been reported to be preserved even in the brain areas severely affected in neurodegenerative diseases including the striatum of Huntington disease, the neocortex and hippocampus of Alzheimer and Pick diseases, and the cerebellum of spinocerebellar degeneration.[15–18] Therefore, the defect in GluR2 Q/R site-editing is not a non-specific phenomena occurring in dying neurons, but is a molecular abnormality relevant to the pathogenesis of sporadic ALS.

DEATH OF MOTOR NEURONS IN OTHER MOTOR NEURON DISEASES

SOD1-associated familial ALS (ALS1) is the most frequent familial ALS,[19] and mutated human SOD1 transgenic animals have been studied extensively as a disease model of ALS1, and sometimes of ALS in general.[20] GluR2 mRNA in the motor neurons of symptom-manifesting $SOD1^{G93A}$ and $SOD1^{H46R}$ transgenic rats was completely edited at the Q/R site.[21] Therefore, the death-inducing molecular mechanisms may be different between sporadic ALS and ALS1. Indeed, disease-causative mutations of the SOD1 gene were found in only a small percentage of

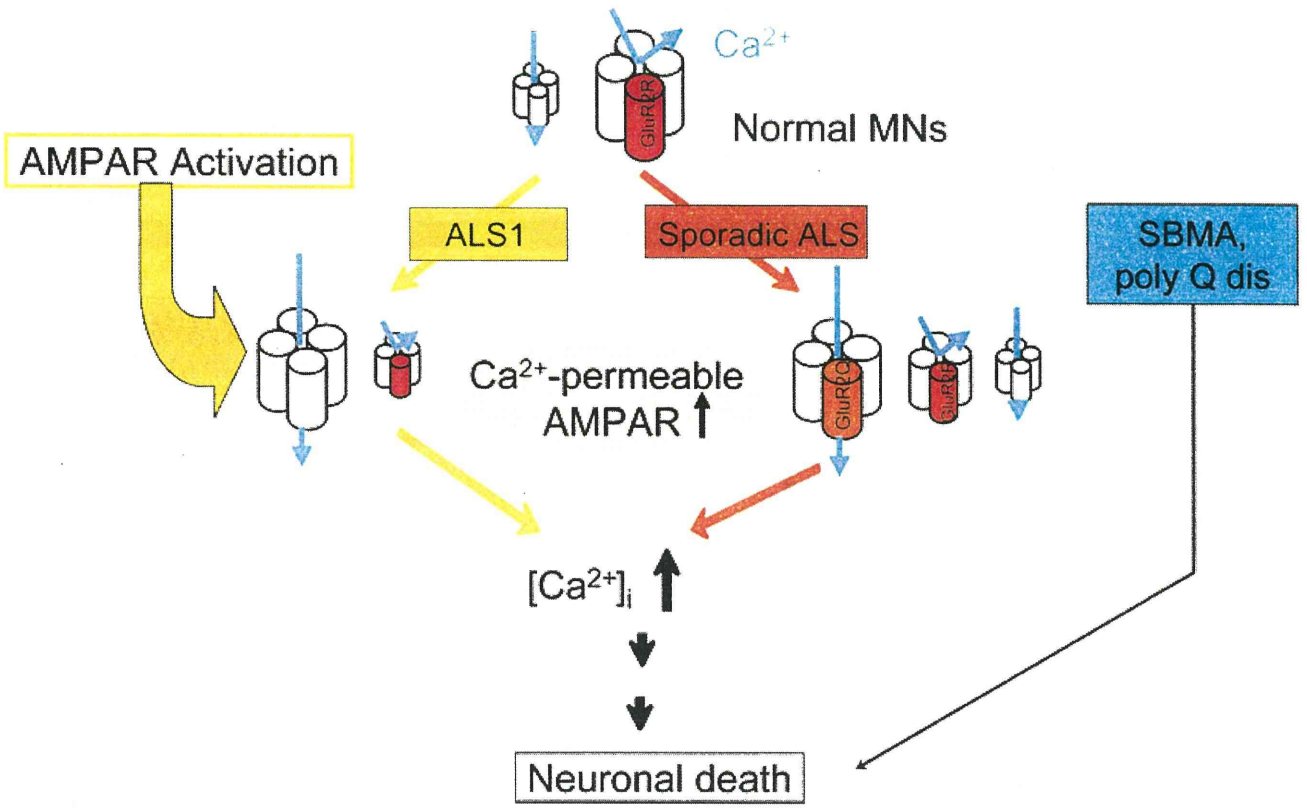


Fig. 2 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated and -unmediated pathways in death of motor neurons: Motor neurons normally express abundant Ca^{2+}-impermeable AMPA receptors and a small proportion of GluR2-lacking Ca^{2+}-permeable AMPA receptors, as well. Motor neurons in sporadic ALS patients express a significant proportion of glutamine/arginine (Q/R) site-unedited GluR2, resulting in an increased of Q/R site-unedited GluR2-containing Ca^{2+}-permeable AMPA receptors. By contrast, motor neurons in SOD1-associated familial ALS (ALS1) patients express greater amounts of GluR2-lacking AMPA receptors than those in control subject due to up-regulation of GluR3 and neuronal death occurs when AMPA receptors are activated. AMPA receptor-mediated neuronal death does not play a role in death of motor neurons in spinal and bulbar spinal atrophy (SBMA), a genomic glutamine codon (CAG)-repeat expansion disease.

patients with sporadic ALS.[22] Furthermore, abnormal accumulation of TDP-43 demonstrated in motor neurons of sporadic ALS[23,24] was not observed in motor neurons of SOD1-associated familial ALS.[25,26]

However, many recent studies support critical roles of Ca^{2+}-permeable AMPA receptors in motor neuron degeneration in ALS1. Transgenic animal studies have recently solidified the link between Ca^{2+}-permeable AMPA receptors and motor neuron loss in ALS1. Specifically, crossing $SOD1^{G93A}$ transgenic mouse models of ALS1 with either mice lacking GluR2 entirely[27] or mice expressing a modified GluR2 gene that encodes aspargine (N) at the Q/R site (GluR-B(N)), which is equivalent to Q/R site-unedited GluR2Q,[28] resulted in marked acceleration of the disease. Conversely, when mice with decreased numbers of Ca^{2+}-permeable AMPA receptors in their motor neurons (via targeted GluR2 overexpression) were crossed with the $SOD1^{G93A}$ transgenic mice, the disease was significantly delayed.[29] Because all the GluR2 mRNA was edited at the Q/R site in ALS1 model rat motor neurons,[21] these lines of evidence may indicate that an increase of Ca^{2+}-permeable AMPA receptors resulted from an increase of GluR2-lacking, but not of Q/R site-unedited GluR2-containing, AMPA receptors in ALS1. On the other hand, an increase in GluR3 mRNA has been reported in the motor neurons of $SOD1^{G93A}$ mice,[30] and the survival of these mice can be prolonged by the administration of GluR3 antisense protein nucleic acid.[31] Similarly, there was an up-regulation of total AMPA receptor subunit mRNAs due to a selective increase of GluR3 mRNA in motor neurons after the initiation of kainic acid infusion.[32]

Therefore, AMPA receptor-mediated neuronal death plays a pivotal role in both sporadic ALS and familial ALS1, but via different molecular mechanisms (Fig. 2). Investigation into the link between abnormal TAR DNA-binding protein of Mr 43 kDa (TDP-43) and GluR2 Q/R site under-editing may promote our understanding about the ALS pathogenesis.

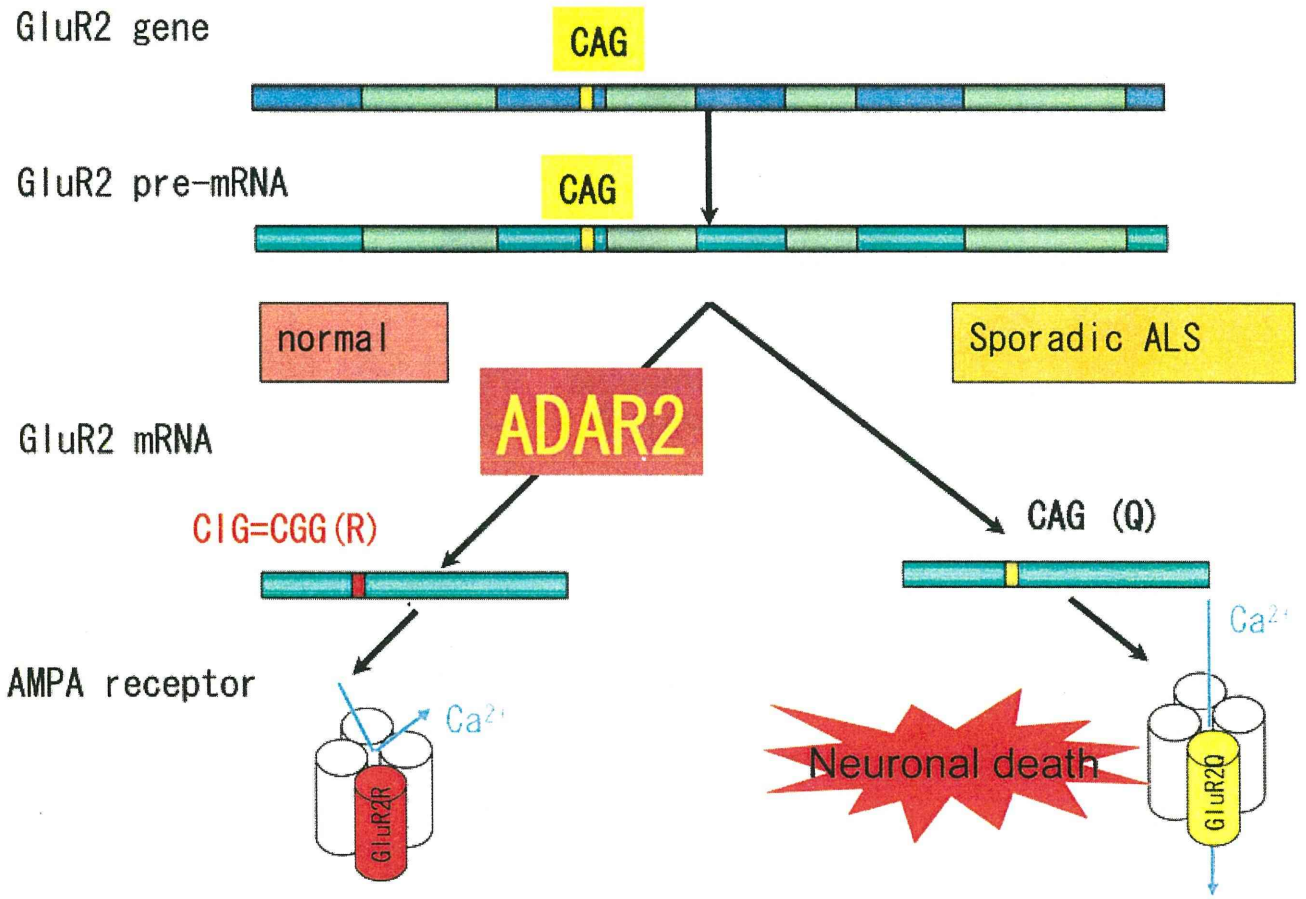


Fig. 3 RNA editing and neuronal death in sporadic ALS. The genomic glutamine codon (CAG) is converted to CIG post-transcriptionally due to A-to-I conversion by ADAR2, which is translated as CGG (arginine; R) in normal neurons. By contrast, due to ADAR2 underactivity, unedited mRNA is translated as GluR2 with glutamine (Q) at the Q/R site in motor neurons of sporadic ALS. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors containing Q/R site-unedited GluR2 are Ca^{2+}-permeable and mediate neuronal death.

Another example of motor neuron disease is spinal and bulbar spinal atrophy (SBMA), in which the CAG-repeat expansion in the androgen receptor gene has been demonstrated[33] and pharmacological castration is therapeutically effective in animal models.[34,35] *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity but not AMPA receptor-mediated neurotoxicity plays a role in the neuronal death in model mice of Huntington disease, another CAG-repeat expansion disease,[36] and we found that GluR2 Q/R site-editing was not affected in dying motor neurons in the autopsied SBMA spinal cord.[21]

Taken together, it is likely that there are multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades (Fig. 2).[37]

ADAR2 AND SPORADIC ALS

Enzymes responsible for the A-to-I conversion have been termed adenosine deaminases acting on RNA (ADARs), and three structurally related ADARs (ADAR1 to ADAR3) have been identified in mammals.[38] ADAR2 recognize the adenosine residue to be edited in the Q/R site of GluR2 through the structure of the duplex that is formed between the editing site and its editing site complementary sequence (ECS), which is located in the adjacent downstream intron of the precursor (pre-) mRNA[39] and catalyzes conversion of A-to-I at the Q/R site of GluR2 mRNA exon 11.

ADAR2 knockout mice died young from status epilepticus while expressing a markedly high proportion of Q/R site-unedited GluR2, but the ADAR2 knockout mice additionally expressed Q/R site-edited GluR2 without ADAR2 (GluR-B^R) and exhibited normal phenotype without developing seizure activity.[40] It is likely therefore that ADAR2-deficiency induces neuronal death via failure to edit the GluR2 Q/R site, which is demonstrated by our newly developed mouse line in which ADAR2 was conditionally targeted in the motor neurons using the Cre/LoxP system. The mutant mice exhibited

ALS-like phenotype via failure to edit the GluR2 Q/R site completely.[41]

One of the determinants of GluR2 Q/R site-RNA editing is the expression level of ADAR2 mRNA in human brain,[42] and the expression level of ADAR2 mRNA was significantly reduced in ventral gray matter of the autopsy-obtained spinal cord of sporadic ALS cases compared with that of control subjects.[43,44] Therefore, it is likely that a reduction in ADAR2 activity causes death of motor neurons via failure to edit efficiently the GluR2 Q/R site in sporadic ALS (Fig. 3).

These lines of evidence suggest that the drugs that up-regulate GluR2 Q/R site-editing may be potential therapeutic tools for sporadic ALS. We developed a modified HeLa cell line that stably expresses half-edited GluR2 pre-mRNA, and using this cell line, we searched for compounds that increase GluR2 Q/R site-editing. We found that several antidepressants have the potency at a concentration of μM orders.[45] Our results suggest that antidepressants have the potency to enhance GluR2 Q/R site-editing by either up-regulating the ADAR2 mRNA expression level or other unidentified mechanisms. It is important to investigate whether these antidepressants could enhance GluR2 Q/R site-editing *in vivo*. To this end, markers representing a wide range of ADAR2 activity may become useful tools for evaluation of the efficacy of therapy aiming at enhancement of ADAR2 activity. Messenger RNA of cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) is ubiquitously expressed and is particularly abundant in the central nervous system, including motor neurons in the spinal cord. Extents of CYFIP2 K/E site-editing are in the range of 30% to 85% in human brain and spinal cord.[46] Therefore, the extent of CYFIP2 K/E site-editing may become an additional marker for ADAR2 activity in neuronal and other types of cells *in vivo*, as well as *in vitro*.[47]

Because it is apparent that the pathogenesis of sporadic ALS is different from familial ALS cases, including ALS1, research based on patient-derived death-inducing molecular abnormalities is absolutely necessary in sporadic ALS. Although little has been elucidated about the precise pathways of death cascade, evidence that ADAR2 underactivity is the specific molecular change seen in autopsied tissues of patients, will provide both new insight into the pathogenesis of sporadic ALS and also novel therapeutic strategies.

ACKNOWLEDGMENT

This study was supported, in part, by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, grants-in-aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan, a grant from The Nakabayashi Trust for ALS Research and a grant from the Japan ALS Association.

REFERENCES

1. Schymick JC, Talbot K, Traynor BJ. Genetics of sporadic amyotrophic lateral sclerosis. *Hum Mol Genet* 2007; **16**: R233–R242.
2. Carriedo SG, Yin HZ, Weiss JH. Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. *J Neurosci* 1996; **16**: 4069–4079.
3. Lu YM, Yin HZ, Chiang J, Weiss JH. Ca^{2+}-permeable AMPA/kainate and NMDA channels: high rate of Ca^{2+} influx underlies potent induction of injury. *J Neurosci* 1996; **16**: 5457–5465.
4. Buckingham SD, Kwak S, Jones AK, Blackshaw SE, Sattelle DB. Edited GluR2, a gatekeeper for motor neurone survival? *Bioessays* 2008; **30**: 1185–1192.
5. Seeburg PH, Single F, Kuner T, Higuchi M, Sprengel R. Genetic manipulation of key determinants of ion flow in glutamate receptor channels in the mouse. *Brain Res* 2001; **907**: 233–243.
6. Seeburg PH. A-to-I editing: new and old sites, functions and speculations. *Neuron* 2002; **35**: 17–20.
7. Burnashev N, Monyer H, Seeburg P, Sakmann B. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* 1992; **8**: 189–198.
8. Hollmann M, Hartley M, Heinemann S. Ca2+ permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 1991; **252**: 851–853.
9. Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 1991; **252**: 1715–1718.
10. Greger IH, Khatri L, Ziff EB. RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. *Neuron* 2002; **34**: 759–772.
11. Greger IH, Khatri L, Kong X, Ziff EB. AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* 2003; **40**: 763–774.
12. Lomeli H, Mosbacher J, Melcher T *et al.* Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* 1994; **266**: 1709–1713.
13. Brusa R, Zimmermann F, Koh D *et al.* Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science* 1995; **270**: 1677–1680.
14. Kawahara Y, Ito K, Sun H, Aizawa H, Kanazawa I, Kwak S. Glutamate receptors: RNA editing and death of motor neurons. *Nature* 2004; **427**: 801.

15. Akbarian S, Smith M, Jones E. Editing for an AMPA receptor subunit RNA in prefrontal cortex and striatum in Alzheimer's disease, Huntington's disease and schizophrenia. *Brain Res* 1995; **699**: 297–304.
16. Paschen W, Hedreen J, Ross C. RNA editing of the glutamate receptor subunits GluR2 and GluR6 in human brain tissue. *J Neurochem* 1994; **63**: 1596–1602.
17. Pellegrini-Giampietro DE, Bennett MV, Zukin RS. AMPA/kainate receptor gene expression in normal and Alzheimer's disease hippocampus. *Neuroscience* 1994; **61**: 41–49.
18. Suzuki T, Tsuzuki K, Kameyama K, Kwak S. Recent advances in the study of AMPA receptors. *Folia Pharmacol Jpn* 2003; **122**: 515–526.
19. Rosen DR, Siddique T, Patterson D *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993; **362**: 59–62.
20. Gurney ME, Pu H, Chiu AY *et al.* Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994; **264**: 1772–1775.
21. Kawahara Y, Sun H, Ito K *et al.* Underediting of GluR2 mRNA, a neuronal death inducing molecular change in sporadic ALS, does not occur in motor neurons in ALS1 or SBMA. *Neurosci Res* 2006; **54**: 11–14.
22. Jackson M, Al-Chalabi A, Enayat ZE, Chioza B, Leigh PN, Morrison KE. Copper/zinc. superoxide dismutase 1 and sporadic amyotrophic lateral sclerosis: analysis of 155 cases and identification of a novel insertion mutation. *Ann Neurol* 1997; **42**: 803–807.
23. Arai T, Hasegawa M, Akiyama H *et al.* TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Com* 2006; **351**: 602–611.
24. Neumann M, Sampathu DM, Kwong LK *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 2006; **314**: 130–133.
25. Mackenzie IR, Rademakers R. The molecular genetics and neuropathology of frontotemporal lobar degeneration: recent developments. *Neurogenetics* 2007; **8**: 237–248.
26. Tan CF, Eguchi H, Tagawa A *et al.* TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta Neuropathol (Berl)* 2007; **113**: 535–542.
27. Van Damme P, Braeken D, Callewaert G, Robberecht W, Van Den Bosch L. GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol* 2005; **64**: 605–612.
28. Kuner R, Groom AJ, Bresink I *et al.* Late-onset motoneuron disease caused by a functionally modified AMPA receptor subunit. *Proc Natl Acad Sci USA* 2005; **102**: 5826–5831.
29. Tateno M, Sadakata H, Tanaka M *et al.* Calcium-permeable AMPA receptors promote misfolding of mutant SOD1 protein and development of amyotrophic lateral sclerosis in a transgenic mouse model. *Hum Mol Genet* 2004; **13**: 2183–2196.
30. Spalloni A, Albo F, Ferrari F *et al.* Cu/Zn-superoxide dismutase (GLY93–>ALA) mutation alters AMPA receptor subunit expression and function and potentiates kainate-mediated toxicity in motor neurons in culture. *Neurobiol Dis* 2004; **15**: 340–350.
31. Rembach A, Turner BJ, Bruce S *et al.* Antisense peptide nucleic acid targeting GluR3 delays disease onset and progression in the SOD1 G93A mouse model of familial ALS. *J Neurosci Res* 2004; **77**: 573–582.
32. Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S. Slow and selective death of spinal motor neurons in vivo by intrathecal infusion of kainic acid: implications for AMPA receptor-mediated excitotoxicity in ALS. *J Neurochem* 2006; **98**: 782–791.
33. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 1991; **352**: 77–79.
34. Katsuno M, Adachi H, Doyu M *et al.* Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. *Nat Med* 2003; **9**: 768–773.
35. Katsuno M, Adachi H, Kume A *et al.* Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Neuron* 2002; **35**: 843–854.
36. Cowan CM, Raymond LA. Selective neuronal degeneration in Huntington's disease. *Curr Top Dev Biol* 2006; **75**: 25–71.
37. Kwak S, Weiss JH. Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. *Curr Opin Neurobiol* 2006; **16**: 281–287.
38. Nishikura K. Editor meets silencer: crosstalk between RNA editing and RNA interference. *Nature Rev Mol Cell Biol* 2006; **7**: 919–931.
39. Higuchi M, Single F, Kohler M, Sommer B, Sprengel R, Seeburg P. RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell* 1993; **75**: 1361–1370.
40. Higuchi M, Maas S, Single FN *et al.* Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* 2000; **406**: 78–81.

41. Hideyama T, Yamashita T, Tsuji S *et al.* Development of a mouse model of sporadic ALS by deficient RNA editing. *Abstr Soc Neurosci* 2008; **745**: 17.
42. Kawahara Y, Ito K, Sun H, Kanazawa I, Kwak S. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. *Eur J Neurosci* 2003; **18**: 23–33.
43. Kawahara Y, Kwak S. Excitotoxicity and ALS: what is unique about the AMPA receptors expressed on spinal motor neurons? *Amyotroph Lateral Scler Other Motor Neuron Disord* 2005; **6**: 131–144.
44. Kwak S, Kawahara Y. Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. *J Mol Med* 2005; **83**: 110–120.
45. Sawada J, Yamashita T, Aizawa H, Aburakawa Y, Hasebe N, Kwak S. Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line. *Neurosci Res* 2009; **64**: 251–258.
46. Nishimoto Y, Yamashita T, Hideyama T, Tsuji S, Suzuki N, Kwak S. Determination of editors at the novel A-to-I editing positions. *Neurosci Res* 2008; **61**: 201–206.
47. Kwak S, Nishimoto Y, Yamashita T. Newly identified ADAR-mediated A-to-I editing positions as a tool for ALS research. *RNA Biol* 2008; **5**: 193–197.

Neurobiology of Disease

Induced Loss of ADAR2 Engenders Slow Death of Motor Neurons from Q/R Site-Unedited GluR2

Takuto Hideyama,[1,2] Takenari Yamashita,[1,2] Takeshi Suzuki,[3] Shoji Tsuji,[2] Miyoko Higuchi,[5] Peter H. Seeburg,[5] Ryosuke Takahashi,[6] Hidemi Misawa,[4] and Shin Kwak[1,2]

[1]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency and [2]Department of Neurology, Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113-8655, Japan, [3]Division of Basic Biological Sciences and [4]Department of Pharmacology, Faculty of Pharmacy, Keio University, Minato-ku, Tokyo 105-8512, Japan, [5]Department of Molecular Neuroscience, Max Planck Institute of Medical Research, 69120 Heidelberg, Germany, and [6]Department of Neurology, Graduate School of Medicine, University of Kyoto, Sakyo-ku, Kyoto 606-8507, Japan

GluR2 is a subunit of the AMPA receptor, and the adenosine for the Q/R site of its pre-mRNA is converted to inosine (A-to-I conversion) by the enzyme called adenosine deaminase acting on RNA 2 (ADAR2). Failure of A-to-I conversion at this site affects multiple AMPA receptor properties, including the Ca^{2+} permeability of the receptor-coupled ion channel, thereby inducing fatal epilepsy in mice (Brusa et al., 1995; Feldmeyer et al., 1999). In addition, inefficient GluR2 Q/R site editing is a disease-specific molecular dysfunction found in the motor neurons of sporadic amyotrophic lateral sclerosis (ALS) patients (Kawahara et al., 2004). Here, we generated genetically modified mice (designated as AR2) in which the ADAR2 gene was conditionally targeted in motor neurons using the Cre/loxP system. These AR2 mice showed a decline in motor function commensurate with the slow death of ADAR2-deficient motor neurons in the spinal cord and cranial motor nerve nuclei. Notably, neurons in nuclei of oculomotor nerves, which often escape degeneration in ALS, were not decreased in number despite a significant decrease in GluR2 Q/R site editing. All cellular and phenotypic changes in AR2 mice were prevented when the mice carried endogenous GluR2 alleles engineered to express edited GluR2 without ADAR2 activity (Higuchi et al., 2000). Thus, loss of ADAR2 activity causes AMPA receptor-mediated death of motor neurons.

Introduction

GluR2 (also known as GluR-B or GluA2) is a subunit of the AMPA receptor. The adenosine within the glutamine codon for the Q/R site of its pre-mRNA is converted to inosine (A-to-I conversion) (Yang et al., 1995) by adenosine deaminase acting on RNA 2 (ADAR2) (Melcher et al., 1996). Because inosine is read as guanosine during translation, the genomic glutamine codon (Q: CAG) is converted to a codon for arginine (R: CIG) at the Q/R site of GluR2 in virtually all neurons in the mammalian brain (Seeburg, 2002). Conversion of Q to R at the Q/R site of GluR2 affects multiple AMPA receptor properties, including the Ca^{2+} permeability of the receptor-coupled ion channel, receptor trafficking, and assembly of receptor subunits (Sommer et al., 1991; Burnashev et al., 1992; Greger et al., 2002, 2003). Genetically modified mice in which the Q/R site of GluR2 remains unedited displayed fatal status epilepticus at early postnatal stages with exaggerated excitation of neurons (Brusa et al., 1995; Feldmeyer et al., 1999). Systemic ADAR2-null mice exhibit a similar phenotype, which was attributed to the absence of GluR2 Q/R site RNA editing (Higuchi et al., 2000). These findings indicate that the A-to-I conversion of the GluR2 Q/R site by ADAR2 is crucial for survival in mice. However, it has not been demonstrated whether neuronal death occurs in mice lacking GluR2 Q/R site editing or in those lacking ADAR2.

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease. Patients with sporadic ALS account for >90% of all cases, and the majority of them do not carry mutations in the causative genes of familial ALS that have been identified thus far (Schymick et al., 2007; Beleza-Meireles and Al-Chalabi, 2009). There is strong evidence indicating that AMPA receptor-mediated excitotoxic mechanism plays a pathogenic role in ALS and SOD1-associated familial ALS model animals (Rothstein et al., 1992; Carriedo et al., 1996; Van Damme et al., 2005). Recently, we demonstrated that a significant proportion of GluR2 mRNA was unedited at the Q/R site in spinal motor neurons of postmortem patients with sporadic ALS. This is in marked contrast to the fact that all GluR2 mRNA was edited in the motor neurons of control subjects (Takuma et al., 1999; Kawahara et al., 2004) and of patients with motor neuron diseases other than sporadic ALS (Kawahara et al., 2006), as well as in dying neurons in other neurodegenerative diseases, including Purkinje cells of patients with spinocerebellar degeneration (Paschen et al., 1994; Akbarian et al., 1995; Kawahara et al., 2004; Suzuki et al., 2003). The disease specificity of inefficient GluR2 Q/R site editing implies the pathogenic relevance of ADAR2 insufficiency in the death of motor neurons in sporadic ALS but

Received April 20, 2010; revised July 2, 2010; accepted July 13, 2010.

This study was supported in part by Ministry of Education, Culture, Sports, Science, and Technology of Japan Grants-in-Aid for Scientific Research 17390251, 19390235, and 20023008 (S.K.), Ministry of Health, Labor, and Welfare of Japan Grant H18-Kokoro-017 (S.K.), and Amyotrophic Lateral Sclerosis Association Grant 875 (P.H.S.). We thank Dr. R. B. Emeson at Vanderbilt University (Nashville, TN) for antibodies to ADAR2 and D. Kimura, K. Awabayashi, Dr. J. Shimizu, Dr. M. Fukaya, and T. Kakinoki for technical assistance.

Correspondence should be addressed to Dr. Shin Kwak, Department of Neurology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kwak-tky@umin.ac.jp.

DOI:10.1523/JNEUROSCI.2021-10.2010

 0270-6474/10/3011917-09$15.00/0

leaves open the possibility that other genes whose products remain unedited by ADAR2 insufficiency might contribute to the demise of motor neurons.

We therefore generated a conditional ADAR2 knock-out mouse strain (designated here as AR2), using the Cre/loxP recombination system, and demonstrated that the loss of ADAR2 activity induces the slow death of motor neurons also in the mouse. Importantly, all motor neuron death in AR2 mice could be prevented by substituting the wild-type GluR2 alleles for alleles point mutated to express Q/R site-edited GluR2 in the absence of ADAR2. Our genetic studies in the mouse clearly demonstrate that the underediting of the GluR2 Q/R site specifically induces death of motor neurons with reduced ADAR2 activity.

Materials and Methods

All studies were performed in accordance with the Declaration of Helsinki, the Guideline of Animal Studies of the University of Tokyo, and National Institutes of Health. The committee of animal handling of the University of Tokyo also approved the experimental procedures used.

ADAR2 ^{flox} *allele and conditional ADAR2 knock-out mice.* DNA for the targeted region was obtained from a mouse strain 129/SvEv genomic library (supplemental Table S1, available at www.jneurosci.org as supplemental material). A LoxP site was inserted into intron 6 and another LoxP site was inserted into intron 9 of the mouse *ADAR2* gene (*adarb1*), along with a selection cassette containing a neomycin resistance gene (Neo) flanked by flippase recognition target (FRT) sites (Fig. 1*A*). Exons 7–9 encode the majority of the adenosine deaminase motif. Chimeric mice were generated by injection of a targeted embryonic stem cell clone into C57BL/6-derived blastocysts. *ADAR2*$^{flox/+}$ intercrosses produced *ADAR2*$^{flox/flox}$ mice at apparent Mendelian frequencies, and *ADAR2*$^{flox/flox}$ homozygous mice were phenotypically normal. Determination of the *ADAR2*^{flox} allele was conducted by genomic PCR (Fig. 1*B*). Then, to knock-out ADAR2 activity selectively in motor neurons, we crossed *ADAR2*$^{flox/flox}$ mice with VAChT–Cre.Fast mice to obtain AR2 mice.

AR2 mice. Intercrosses of *ADAR2*$^{flox/+}$/VAChT–Cre.Fast mice produced *ADAR2*$^{flox/flox}$/VAChT–Cre.Fast (AR2) mice, either heterozygous or homozygous for the Cre transgene, which directs restricted Cre expression under the control of the vesicular acetylcholine transporter gene promoter in a subset of cholinergic neurons, including the spinal motor neurons (Misawa et al., 2003). Cre expression levels were found not to differ in mice heterozygous or homozygous for the VAChT–Cre.Fast transgene (Misawa et al., 2003). The same intercrosses also produced, as littermates of AR2, *ADAR2*$^{flox/flox}$ (Ctl1) and *ADAR2*$^{+/+}$/VAChT–Cre-.Fast mice (Ctl2), which were used as controls. Both genders of AR2 and control mice were used, but littermates heterozygous for the floxed ADAR2 allele were not used in this study. All genotyping was performed by PCR on DNA from tail biopsies. PCR primers and amplicon sizes for the different alleles are listed in supplemental Table S1 (available at www.jneurosci.org as supplemental material).

AR2/GluR-B$^{R/R}$ *mice.* AR2/GluR-$B^{R/R}$ mice were generated by intercrossing ADAR2$^{flox/-}$/VAChT–Cre.Fast/GluR-$B^{R/-}$mice, which had been produced by crossbreeding AR2 mice with GluR-$B^{R/R}$ mice. The AR2/GluR-$B^{R/R}$ mice used by us were either heterozygous or homozygous for the Cre transgene (Misawa et al., 2003) and homozygous for the floxed ADAR2 and the GluR-B(R) allele. The desired genotype was found approximately once in every 20 offspring. Other genotypes produced by the intercrosses were not used in this study. All genotyping for the ADAR2 and GluR2 (GluR-B) alleles as well as for the Cre transgene was by PCR on DNA extracted from tail biopsies. PCR primers and amplicon sizes for the different alleles are listed in supplemental Table S1 (available at www.jneurosci.org as supplemental material).

Genomic PCR and reverse transcription-PCR. Genomic DNA was extracted from mouse tails using the High Pure PCR Template Preparation kit (Roche). Total RNA was isolated from brain and spinal cord tissue, and first-strand cDNA was synthesized and then treated with DNase I (Invitrogen) as described previously (Kawahara et al., 2003b). Primer pairs and the conditions used for PCR are presented in supplemental Table S1 (available at www.jneurosci.org as supplemental material). Positions of primer pairs used for genomic ADAR2 PCR (Fig. 1*A*, F1/R1) and ADAR2 reverse transcription (RT)-PCR (Fig. 1*C*, F2/R2) are indicated.

Analysis for editing efficiency at A-to-I sites. Editing efficiencies at the Q/R sites in GluR2 mRNAs were calculated by quantitative analyses of the digests of RT-PCR products with BbvI as described previously (Takuma et al., 1999; Kawahara et al., 2003a, 2004). In brief, 2 μl of cDNA were subjected to first PCR in duplicate in a reaction mixture of 50 μl containing 200 mM each primer, 1 mM dNTP Mix (Eppendorf), 5 μl of 10× PCR buffer, and 1 μl of Advantage 2 Polymerase mix (Clontech). The PCR amplification began with a 1 min denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 68°C for 40 s. Nested PCR was conducted on 2 μl of the first PCR product under the same conditions with the exception of the annealing temperature (58°C). Primer pairs used for each PCR were listed in supplemental Table S1 (available at www.jneurosci.org as supplemental material). After gel purification using the Zymoclean Gel DNA Recovery kit according to the protocol of the manufacturer (Zymo Research), an aliquot (0.5 mg) was incubated with BbvI (New England Biolabs) at 37°C for 12 h. The PCR products originating from Q/R site-edited GluR2 mRNA had one intrinsic restriction enzyme recognition site, whereas those originating from unedited mRNA had an additional recognition site. Thus, restriction digestion of the PCR products originating from edited GluR2 mRNA should produce different numbers of fragments (two bands at 219 and 59 bp) from those originating from unedited GluR2 mRNA (three bands at 140, 79, and 59 bp). Because the 59 bp band would originate from both edited and unedited mRNA but the 219 bp band would originate from only edited mRNA, we quantified the molarity of the 219 and 59 bp bands using the 2100 Bioanalyzer (Agilent Technologies) and calculated the editing efficiency as the ratio of the former to the latter for each sample (supplemental Table S1, available at www.jneurosci.org as supplemental material).

With similar methods, we calculated the editing efficiencies at the Q/R sites in GluR5 and GluR6 mRNA and in GluR2 pre-mRNA, the R/G site in GluR2 mRNA, and the I/V site in Kv1.1 mRNA (Paschen et al., 1994; Takuma et al., 1999; Kawahara et al., 2003a, 2004; Nishimoto et al., 2008). The following restriction enzymes were used for restriction digestion of the respective A-to-I sites: BbvI for the Q/R sites, MfeI (New England Biolabs) for the I/V site, and MseI (New England Biolabs) for the R/G site. Primer pairs used for each PCR and sizes of restriction digests of PCR products were indicated in supplemental Table S1 (available at www.jneurosci.org as supplemental material).

Behavioral analyses. Using a mouse-specific rotarod (SN-445; Neuroscience Corp.), we determined the maximal time before falling at 10 rpm during a 180 s period; each run consisted of three trials. Grip strength was measured with a dynamometer (NS-TRM-M; Neuroscience Corp.). Measurements were conducted weekly by a researcher blind to genotype and age of the mice.

Isolation of single motor neurons and brain tissue. Single-cell isolation from frozen spinal cord tissue was performed with a laser microdissection system (Leica AS LMD; Leica Microsystems) as described previously (Kawahara et al., 2003b, 2004). All of the large motor neurons (diameter larger than 20 μm) in the anterior horn were dissected from 14-μm-thick cervical cord sections, and three neurons each were collected together into respective single test tubes containing 200 μl of TRIZOL Reagent. In addition, using the same method, nuclei of oculomotor nerve and of facial nerve were dissected from the brainstem sections of AR2 mice and control mice at 12 months of age. The positions of these cranial nerve nuclei were identified using the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). All samples were kept at −20°C until use.

Immunohistochemistry. Under deep anesthesia with isoflurane, mice were transcardially perfused with 3% paraformaldehyde and 1% glutaraldehyde in PBS. The brains and spinal cords were removed and immersed in serially increasing concentrations of a sucrose–PBS solution (final sucrose concentration of 30%). The immunohistochemical procedure was performed on 10-μm-thick sections, which were cut with a cryostat (model HM500 O; Microm). The sections were analyzed with a

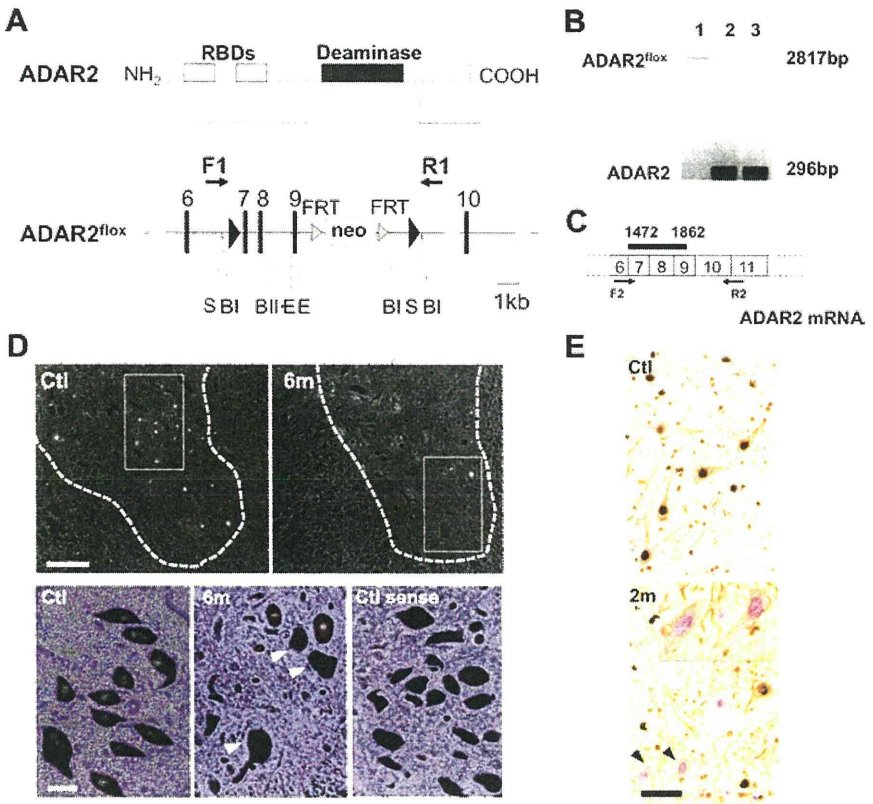


Figure 1. Generation of a conditional ADAR2 knock-out mouse. ***A***, A LoxP site (filled triangle) was inserted into intron 6 and another LoxP site in intron 9 with a selection cassette containing the gene for neomycin resistance (Neo) flanked by FRT sites. Exons are depicted as black bars with numbers. RBDs, RNA binding domains; F1/R1, primer pair (supplemental Table S1, available at www.jneurosci.org as supplemental material) for ***B***; S, SfiI; BI, BglI; BII, BglII; E, EcRI. ***B***, Genomic PCR using template DNA obtained from the tails of *ADAR2*$^{flox/flox}$ mice (lane 1), *ADAR2*$^{flox/+}$ mice (lane 2), and *ADAR2*$^{+/+}$ mice (lane 3). ***C***, Exons excised by recombination are shown as shaded areas in the mRNA, and a black bar indicates the *in situ* hybridization probe (supplemental Table S1, available at www.jneurosci.org as supplemental material) for ***D***. F2/R2, Primer pair (supplemental Table S1, available at www.jneurosci.org as supplemental material) used in Figure 2 *B*. ***D***, *In situ* hybridization using a probe that encompasses the region excised by Cre-mediated recombination. There is a large number of punctate signals in the gray matter (outlined with dotted lines) of control mice (Ctl), whereas nuclei of some large neurons in the anterior horn were devoid of signal in the *ADAR2*$^{flox/flox}$/VAChT–Cre.Fast (AR2) mice at 6 months of age (6m; arrowheads in magnified view). The sense probe did not yield a visible signal in the control mice at the same age (Ctl sense). Scale bars: top panels, 200 μm; bottom panels, 25 μm. ***E***, All SMI-32-positive large neurons in the anterior horn (AHCs, brown color in the cytoplasm) of the cervical cord (C5) were ADAR2 positive (dark gray color in the nuclei) in the control mice (Ctl), whereas some of them were devoid of ADAR2 immunoreactivity in AR2 mice at 2 months of age (2m, arrowheads and inset). Sections were counterstained with hematoxylin. Scale bar: 50 μm; inset, 25 μm.

standard avidin–biotin–immunoperoxidase complex method using the M.O.M. Immunodetection kit (Vector Laboratories) for mouse primary antibodies and Vectastain ABC IgGs (Vector Laboratories) for other primary antibodies. The following primary antibodies were used: mouse anti-nonphosphorylated neurofilament H (SMI-32; dilution at 1:1000; Covance), mouse anti-neuronal nuclei (NeuN) (dilution at 1:500; Millipore Bioscience Research Reagents), sheep anti-rat RED1 (ADAR2) N terminus [dilution at 1:500; a gift from Dr. R. B. Emeson (Sansam et al., 2003)], rabbit anti-glial fibrillary acidic protein (GFAP) (dilution at 1:200; Lab Vision), and rat anti-mouse MAC-2 (dilution at 1:500; Cedarlane). Color was developed with the HRP–DAB System (Vector Laboratories).

Muscles and neuromuscular junctions. Medial gastrocnemius muscles and medial quadriceps muscles were dissected, pinned in mild stretch, and mounted on cork blocks and were quickly frozen in isopentane–liquid nitrogen. Samples were stored at −80°C until use. Five-micrometer-thick transverse frozen sections were stained with hematoxylin and eosin. Twenty-micrometer-thick frozen longitudinal sections were stained with tetramethylrhodamine–bungarotoxin. The same section was incubated with monoclonal antibodies to neurofilament (NF160; dilution at 1:200; Millipore Bioscience Research Reagents) and synaptophysin (dilution at 1:100; Cell Signaling Technologies) and then with Alexa Fluor 488 rabbit anti-mouse IgG (dilution at 1:100; Invitrogen) as the secondary antibody. Stained sections were examined under an LSM-510 confocal microscope system (Carl Zeiss).

Electrophysiology. Mice were anesthetized with isoflurane and placed in a prone position on a thermal pad at 37°C for the examination. Electromyogram (EMG) recordings using a Power Lab 26T and EMG machine (AD Instruments) were obtained using a 29-gauge, Teflon-coated, monopolar needle electrode. The recording electrode was inserted into the gastrocnemius muscles, and spontaneous electrical activity was recorded for 120 s using a Lab Chart analysis system (AD Instruments).

Morphological observation and stereology. Sections of the fifth cervical (C5) and fifth lumbar (L5) spinal cord segments were sequentially immunostained with RED1 and SMI-32 using the HRP–DAB system with and without the addition of $NiCl_2$ for color development. Some sections were immunostained with NeuN. ADAR2-positive and -negative neurons were separately counted among SMI-32-positive neurons with diameters larger than 20 μm in 10 sections for each mouse. The number of NeuN-positive neurons with diameter smaller than 20 μm in the ventral gray matter (ventral to the line running though the ventral edge of the central canal) was counted in 10 C5 sections for each mouse at 12 months of age. None of the NeuN-positive small neurons exhibited SMI-32 or GFAP immunoreactivity. The entire brainstem of each mouse at 12 months of age was cut axially to produce a 10-μm-thick section, and the numbers of all the neurons with nucleoli in the nuclei of cranial motor nerves were counted under a light microscope after cresyl violet staining. The position of each nucleus was stereologically determined using a mouse brain atlas (Paxinos and Franklin, 2001). The positions from the bregma were from −3.80 to −4.24 mm (nucleus of oculomotor nerve), from −4.36 to −4.48 mm (nucleus of trochlear nerve), from −4.84 to −5.34 mm (motor nucleus of trigeminal nerve), from −5.52 to −5.80 mm (nucleus of abducens nerve), from −5.68 to −6.48 mm (nucleus of facial nerve), from −7.08 to −7.92 mm (dorsal nucleus of vagus nerve), and from −7.08 to −8.12 mm (nucleus of hypoglossal nerve). The density of neurons in each nucleus was estimated by dividing the total number of neurons in each nucleus by the volume of the nucleus, which was calculated as the product of the area of the nucleus and the thickness of each section. In addition, transverse, 1-μm-thick, Epon-embedded sections of the anterior horns of the spinal cord, and the ventral roots at the L5 level were prepared and stained with 0.1% toluidine blue. Cell counting was performed by researchers who were blind to the genotype of the mouse.

In situ *hybridization.* Anesthetized mice were perfusion fixed with Tissue Fixative (GenoStaff). Dissected cervical cord tissues were sectioned after they were embedded in paraffin. Antisense and sense *adarb1* cRNA probes (Fig. 1*C*) (supplemental Table S1, available at www.jneurosci.org as supplemental material) were generated from the mouse *adarb1* open reading frame sequence, which was cloned into the pGEMT-Easy vector (Promega). Digoxigenin-labeled cRNA probes were prepared with the DIG RNA Labeling mix (Roche Applied Science). Color was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, and tissue sections were counterstained with Kernechtrot stain solution

(Muto Pure Chemicals). After mounting, 24-bit color images were acquired by scanning of the sections. Digoxigenin signals were isolated by uniformly subtracting the counterstaining color component using Photoshop version 9.0.2 (Adobe Systems) (Ohmae et al., 2006; Takemoto-Kimura et al., 2007).

Statistics. Differences in behavior and survival rates between groups were analyzed using log-rank analysis with SPSS software (version 15; SPSS Inc.), and GraphPad Prism version 4 (GraphPad Software), respectively. The differences in neuronal number between each group and the control samples were examined with a repeated-measures ANOVA. The SPSS version 15 software was used for ANOVA, followed by a Tukey–Kramer statistical test.

Results

Generation of the *ADAR2$^{flox/flox}$*/VAChT–Cre mouse, designated as AR2 mouse

We constructed the mouse *ADAR2^{flox}* allele by flanking exons 7–9 of the *adarb1* gene (mouse *ADAR2* gene) with loxP sites (Fig. 1*A*) (supplemental Table S1, available at www.jneurosci.org as supplemental material). Exons 7–9 encode the majority of the adenosine deaminase motif in the *adarb1* gene (Feng et al., 2006), and Cre-mediated deletion of this region ablates ADAR2 activity. To ablate ADAR2 activity selectively in motor neurons, we crossed *ADAR2$^{flox/flox}$* mice with VAChT–Cre.Fast mice. In VAChT–Cre.Fast mice, Cre expression is under the control of the vesicular acetylcholine transporter gene promoter, which is active in cholinergic neurons, including spinal motor neurons (Misawa et al., 2003). In these transgenic mice, Cre expression is developmentally regulated, and ~50% of motor neurons express Cre by 5 weeks of age, independent of the heterozygous or homozygous state of the transgene (Misawa et al., 2003). The resulting *ADAR2$^{flox/flox}$*/VAChT–Cre.Fast mice, referred to here as AR2 mice (for breeding, see Materials and Methods), therefore would lack ADAR2 activity in a subset of motor neurons in the spinal cord and other brain motor nuclei after expression of Cre by 5 weeks of age. *In situ* hybridization with a probe encompassing the sequence excised by Cre-mediated recombination (Fig. 1*C*) demonstrated that several large neurons in the anterior horn (AHCs) were devoid of *adarb1* gene signal in the AR2 mice, whereas all the AHCs exhibited the signal in control littermates (Fig. 1*D*). Similarly, a subset of the AHCs were devoid of ADAR2 immunoreactivity in AR2 mice, whereas all AHCs exhibited ADAR2 immunoreactivity in the controls (Fig. 1*E*). There was no difference in the results on male and female AR2 mice.

ADAR2 activity in ADAR2-null motor neurons

Next we examined the effects of recombination of the *ADAR2^{flox}* allele on ADAR2 activity. We dissected all large neurons in the anterior horn (AHCs) (for AHC identification, see supplemental Fig. S1*A*, available at www.jneurosci.org as supplemental material) from frozen sections from 2-month-old AR2 mice ($n = 4$) using a laser microdissector (Fig. 2*A*). We verified that these AHCs, but not small neurons in the anterior horn, are the spinal motor neurons by RT-PCR for choline acetyltransferase on a single-cell lysates (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Because RT-PCR of GluR2 mRNA on the lysates of three neurons, but not the lysates of one or two motor neurons, reproducibly yielded amplification products, we analyzed the extent of GluR2 Q/R site editing on RNA extracted from the lysates of three pooled AHCs (designated as a specimen) by quantitative analysis of the BbvI-restriction digests of the RT-PCR products, as described previously (Kawahara et al., 2003b, 2004). Among 116 specimens examined, eight showed 0% and 42 showed 100% Q/R site editing, with the remaining 66 specimens distributed between the ranges of 17 and 98% (Fig. 2*A*) (supplemental Table S2, available at www.jneurosci.org as supplemental material). Because AHCs of control littermates (these carried wild-type ADAR2 alleles or no Cre transgene; see Materials and Methods) expressed only edited GluR2 mRNA, the presence of samples exhibiting 0% Q/R site editing suggests that ADAR2-expressing neurons expressed only edited GluR2 mRNA, whereas ADAR2-null neurons expressed only unedited GluR2 mRNA. Then, DNA and total RNA from the specimens were collected in four different groups according to the proportions of unedited GluR2 (Fig. 2*A*). Using PCR, we demonstrated that the samples with 100% editing efficiency (group 0:3) harbored the truncated *ADAR2^{flox}* gene and Cre transcripts, whereas the samples with 100% editing efficiency (group 3:0) carried the full-length *ADAR2^{flox}* gene and did not express Cre (Fig. 2*B*). Those samples with both edited and unedited GluR2 mRNA (groups 1:2 and 2:1) exhibited both full-length and truncated ADAR2 along with the Cre transcript. These qualitative results are consistent with the assumption that recombination of the *ADAR2^{flox}* alleles occurred in a Cre-dependent manner and that this recombination abolished the editing of the GluR2 Q/R site. Among other A-to-I sites examined, we found a significant reduction in editing efficiency only at the GluR6 Q/R site (supplemental Table S3, available at www.jneurosci.org as supplemental material).

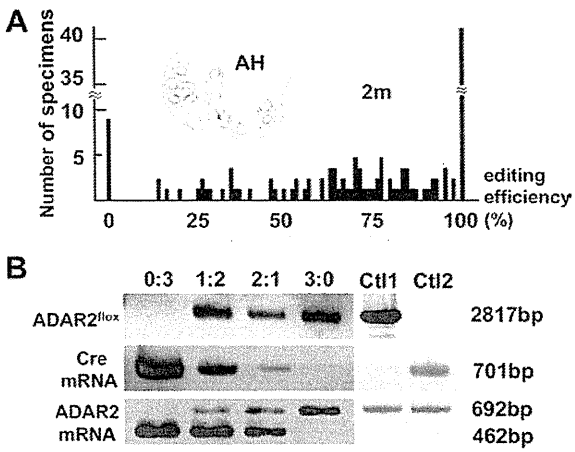


Figure 2. Cre-dependent targeting of ADAR2 and GluR2 Q/R site-editing in motor neurons. ***A***, Frequency histogram of editing efficiency at the GluR2 Q/R site in specimens (lysates containing 3 motor neurons) obtained from AR2 mice at 2 months of age (2m; $n = 4$). Neurons were dissected with a laser microdissector (inset). ***B***, Specimens ($n = 116$) were collected into four groups depending on the predicted number of ADAR2-deficient neurons in each specimen; the groups of specimens containing 3, 2, 1, and 0 unedited GluR2-expressing neurons were designated as groups 0:3, 1:2, 2:1, and 3:0, respectively. The *ADAR2^{flox}* gene and transcripts of the *Cre* gene and the *ADAR2^{flox}* alleles before and after recombination were analyzed for each group by PCR. AHCs expressing unedited GluR2 mRNA (group 0:3) harbored the truncated *ADAR2^{flox}* gene and Cre transcripts, whereas AHCs expressing edited GluR2 mRNA (group 3:0) carried the full-length *ADAR2^{flox}* gene and did not express Cre. Ctl1, *ADAR2$^{flox/flox}$* mice; Ctl2, VAChT–Cre.Fast mice; AH, anterior horn of the spinal cord.

Behavioral changes

AR2 mice were hypokinetic (supplemental movie, available at www.jneurosci.org as supplemental material) and abnormal in posture (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material), but they displayed no overt paralysis or vesico-urinary disturbances and exhibited a normal withdrawal response to noxious stimuli. They showed a lower rotarod performance than their control littermates after 5 weeks of age

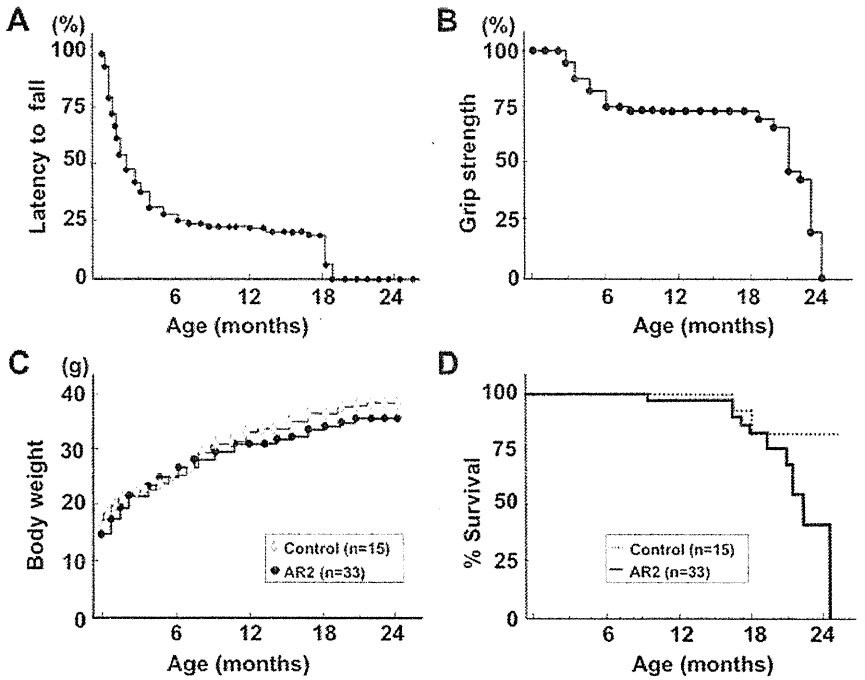


Figure 3. Behavioral changes in AR2 mice. ***A***, Rotarod performance presented as latency to fall (at 10 rpm, 180 s at the maximum) began to decline at 5 weeks of age in AR2 mice and rapidly fell to low levels during the initial 5–6 months, remaining stable until 18 months of age. Control mice exhibited full performance (180 s) until ~12 months of age, followed by slightly lower performance (>164.5 ± 6.4 s) until 24 months. ***B***, Grip strength measured declined with kinetics similar to those of rotarod performance. In ***A*** and ***B***, the scores obtained for the AR2 mice (mean ± SEM; $n = 28$) are indicated as percentage performance of control mice ($n = 15$). ***C***, AR2 mice exhibited slightly lower body weight than controls ($p > 0.05$). ***D***, AR2 mice ($n = 33$) had long lifespans, but the rate of death increased after month 18. The median ± SEM survival was 81.5 ± 16.4 weeks for AR2 mice compared with 105.1 ± 13.5 weeks for control mice ($p = 0.0262$, log-rank analysis).

(Fig. 3*A*), when the Cre expression reached the maximum level (~50% of motor neurons) (Misawa et al., 2003). Their rotarod performance rapidly declined during the initial 5–6 months of life, followed by stable performance until about 18 months of age (Fig. 3*A*). Control mice exhibited full performance (180 s) until ~12 months of age, followed by slightly lower performance (>164.5 ± 6.4 s) until 24 months. Grip strength declined with kinetics similar to those of rotarod performance (Fig. 3*B*). The AR2 mice had slightly lower body weight than the controls (Fig. 3*C*) and were relatively long-lived (81.5 ± 16.4 weeks; mean ± SEM), although not as long as control mice (105.1 ± 13.5 weeks; $p = 0.0262$, log-rank analysis) (Fig. 3*D*).

Pathological alterations in the spinal cords and muscles

Immunohistochemical examination demonstrated that all the AHCs in the spinal cord that were immunoreactive to anti-phosphorylated neurofilament antibodies (SMI-32) showed intense ADAR2 immunoreactivity in their nuclei in control mice, whereas a fraction of these cells was devoid of ADAR2 immunoreactivity in AR2 mice (Fig. 1*E*) (supplemental Fig. S2*B*, available at www.jneurosci.org as supplemental material). There were a number of degenerating AHCs with cytoplasmic vacuoles (Fig. 4*A*) and darkly stained degenerating axons in the ventral roots (Fig. 4*B*). The number of AHCs in AR2 mice markedly decreased between 1 and 2 months of age and then slowly decreased beyond 1 year of age (Fig. 4*C*). The number of ADAR2-positive AHCs in the AR2 mice decreased from 83 to 54% of the number of total AHCs in the age-matched control littermates between 1 and 2 months of age. The rapid reduction in the proportion of ADAR2-positive AHCs during this period is likely attributable to the Cre-dependent recombination of the floxed ADAR2 alleles, because the number of Cre-expressing AHCs in VAChT–Cre-.Fast mice increases developmentally until 5 weeks of age (Misawa et al., 2003). After 2 months of age, the number of ADAR2-positive AHCs did not change over the course of more than 1 year, whereas that of total AHCs decreased from 80 to 54% of the number of AHCs in the age-matched control mice (Fig. 4*C*) (Table 1). Consistent with the Cre-dependent recombination, the proportion of ADAR2-lacking AHCs in AR2 mice is in accordance with that of Cre-expressing AHCs presented in the original study of VAChT–Cre mice (Misawa et al., 2003). Concomitant with AHC degeneration, the number of myelinated axons in the ventral roots was significantly decreased (Table 1).

The kinetics of neuronal loss (Fig. 4*C*) were consistent with the kinetics of progressive motor-selective behavioral deficits (Fig. 3*A*,*B*). The long survival with hypoactivity beyond 6 months of age indicates that the remaining ADAR2-expressing neurons functioned normally during the remainder of life. The high rate of death after 18 months may reflect the failure of the remaining AHCs to compensate for an age-related decline in skeletal muscle power, including a decline in respiratory muscle strength.

We also examined denervation of skeletal muscles. Electromyography performed on AR2 mice at 12 months of age revealed fibrillation potentials and fasciculations, which are common findings in ALS, indicative of muscle fiber denervation and motor unit degeneration and regeneration (Fig. 4*D*). We observed characteristics of denervation, including muscle fiber atrophy, centrally placed nuclei, and pyknotic nuclear clumps in the skeletal muscles of AR2 mice (Fig. 4*E*). Some neuromuscular junctions (NMJs) were not innervated and other NMJs were innervated by ramified axons that innervated more than one NMJ in AR2 mice, indicating reinnervated NMJs (Fig. 4*F*). In contrast, in control mice, all the NMJs were innervated by a single axon. The proportion of denervated NMJs decreased, whereas reinnervated NMJs increased with age in AR2 mice (Fig. 4*F*). In addition, proliferation of activated astrocytes with increased GFAP immunoreactivity and of MAC2-positive activated microglial cells was detected in the anterior horns of AR2 mice (Fig. 4*G*,*H*). These results suggest that degeneration of ADAR2-lacking AHCs induced degeneration of their axon terminals, and then denervated NMJs were reinnervated by collaterally sprouted axons of ADAR2-expressing AHCs after longer survival.

Neurons in the motor nuclei of cranial nerves

The numbers of large neurons in facial and hypoglossal nerve nuclei in AR2 mice were significantly smaller than those in control mice at 12 months of age, whereas the numbers of neurons in nuclei of oculomotor nerves were not decreased (Table 1). Conversely, GluR2 Q/R site editing was significantly decreased both the in oculomotor nerve nuclei (the efficiency of GluR2 Q/R site