ニット全体に占める比率が，他のニューロンに比べて，低く[35,53]，もともとCa^{2+}透過性AMPA受容体の割合が多いためにRNA編集低下の影響を受けやすいことがあげられる．また，これまでのADAR2ノックアウトマウスの研究から，ADAR2活低下がGluR2 Q/R部位の編集異常を通じて神経細胞死の直接原因になり得ること[54]が明らかにされている．ADAR2活性を規定する因子の一つはmRNA発現レベルであり[55,56]，孤発性ALS前角組織では正常対照に比し，ADAR2mRNA発現量が低く[16]，ALS脊髄運動ニューロンではADAR2の酵素活性が低下していることがGluR2 Q/R部位RNA編集異常の原因と考えた．この仮説を証明するために，私たちのグループはADAR2の解析を進めている．

D. ALSの治療に向けて

前述のように孤発性ALSの疾患病態と直接かかわっていると考えられるAMPA受容体関連の分子異常が見つかり，発症メカニズムに基づいた分子標的治療法を開発できる可能性が高まってきた．運動ニューロン選択的にGluR2 Q/R部位のRNA編集を回復できれば，ALSの治療へとつながるものと考えられる．我々は前述の仮説に合致する事実を次々と明らかにしているが，なぜALS

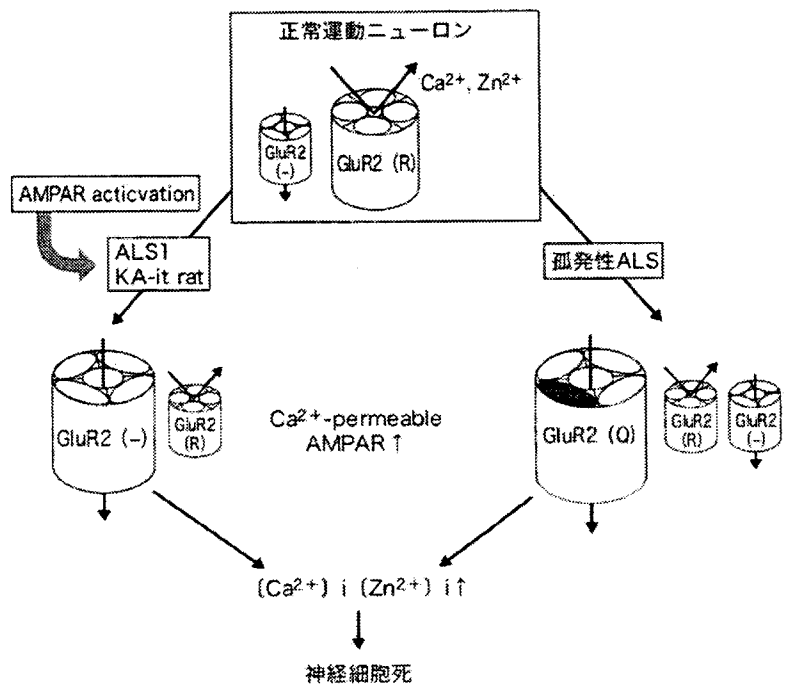


図3　AMPA受容体を介する運動ニューロンの神経細胞死の機序のまとめ（文献52を改変）
正常運動ニューロンではほとんどのAMPA受容体（AMPAR）は編集型GluR2（R）でありCa^{2+}を通さない．わずかながら運動ニューロンでGluR2を含まないCa^{2+}透過性の高いAMPARが存在することが知られている．本文中で述べたように孤発性ALS，ALS1のいずれにもAMPARを介した細胞死のメカニズムのエビデンスがあるが，両者のメカニズムは異なっている．孤発性ALSでは未編集型GluR2（Q）が増加することで透過性AMPARが増加し，一方でALS1ではGluR2の割合の減少により編集型GluR2を含まないAMPA受容体の割合が増加することで細胞内Ca^{2+}濃度が上昇し，神経細胞死が引き起こされる．ただし，前者が単独で神経細胞死が生じるのに対して，後者はSOD1の細胞毒性などの因子が加わる必要がある．

の運動ニューロンで選択的にADAR2活性が低下するのかを含め孤発性ALSの病態メカニズム解明が治療に結びつけられる成果が期待される.

文献

1) Kawahara Y, Ito K, Sun H, et al. RNA editing and death of motor neurons. Nature. 2004; 427: 801.
2) 日出山拓人, 河原行郎, 郭　伸. ALSとAMPA受容体. 脳神経. 2005; 57: 585-98.
3) 郭　伸. ALSの運動ニューロン死とグルタミン酸受容体の分子変化. 神経進歩. 2006; 50(60): 902-11.
4) 五嶋義郎. グルタミン酸受容体の歴史とその背景. Clin Neurosci. 2006; 24(2): 142-4.
5) Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann Neurol. 1986; 19: 105-11.
6) 相澤仁志, 中村良司, 郭　伸. 実験的遅発性興奮性運動ニューロン死. Clin Neurosci. 1998; 16(8): 58-62.
7) 郭　伸. 興奮性アミノ酸と神経障害-神経疾患の実験動物モデル. In: 後藤文男, 他, 編. AnnaulReview神経 1992. 東京: 中外医学社; 1992. p. 15-30.
8) Rothstein JD, Jin L, Dykes-Hoberg M, et al. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc. Natl Acad Sci U S A. 1993; 90: 6591-5.
9) Hirata A, Nakamura R, Kwak S, et al. AMPA receptor-mediated slow neuronal death in the rat spinal cord induced by long-term blockade of glutamate transporters with THA. Brain Res. 1997; 771: 37-44.
10) Carriedo SG, Yin HZ, Weiss JH. Motor neurons are selectively vulnerable to AMPA/kainite receptor-mediated injury in vitro. J Neurosci. 1996; 16: 4069-79.
11) Nakamura R, Kamakura K, Kwak S. Late-onset selective neuronal damage in the rat spinal cord induced by continuous intrathecal administration of AMPA. Brain Research. 1994; 654: 279-85.
12) Sun H, Kawahara Y, Ito K, et al. 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: 782-91.
13) 鈴木岳之, 都築馨介, 亀山仁彦, 他. AMPA受容体の生理機能-受容体機能発現から疾患まで-日薬理誌. 2003; 122: 515-26.
14) 小澤瀞司. 中枢神経系のグルタミン酸受容体. 脳神経. 2001; 53: 605-15.
15) Vennekens R, Voets T, Bindels RJ, et al. Current understanding of mammalian TRP homologues. Cell Calcium. 2002; 31: 253-64.
16) Kawahara Y, Kwak S. Excitatotoxicity and ALS: What is unique about the AMPA receptors expressed on spinal motor neurons ? Amyotrophic lateral sclerosis. 2005; 1-14.
17) 日出山拓人, 河原行郎, 郭　伸. 筋萎縮性側索硬化症の分子病理～病態と治療～. 最新医学. 2005; 60(5): 1072-82.
18) 日出山拓人, 河原行郎, 郭　伸. 筋萎縮性側索硬化症の研究の進歩. 医学のあゆみ. 2005; 212(10): 2613-20.
19) Kwak S, Kawahara Y. Deficient RNA editing of GluR2 and neuronal death in ALS. J Mol Med. 2005; 83: 110-20.
20) 崎村建司. AMPA型グルタミン酸受容体の構造と機能. Clin Neurosci. 2006; 24(2): 145-8.
21) Hollmann M, Hartley M, Heinemann S. Ca^{2+} permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. Science. 1991; 252: 851-3.
22) Verdoorn TA, Burnashev N, Monyer H, et al. Structural determinants of ion flow through recombinant glutamate receptor channels. Science. 1991; 252: 1715-8.
23) Burnashev N, Khodorova A, Jonas P, et al. Calcium-permeable AMPA-kainate receptors in fusiform cerebellar glial cells. Science. 1992; 256: 1566-70.
24) Nutt S, Kamboj R. Differential RNA editing efficiency of AMPA receptor subunit GluR-2 in human brain. Neuroreport. 1994; 5: 1679-83.
25) Geiger JR, Melcher T, Koh DS, et al. Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron. 1995; 15: 193-204.
26) Higuchi M, Single FN, Kohler M, et al. RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. Cell. 1993; 75: 1361-70.
27) Sommer B, Köhler M, Sprengel R, et al. RNA editing in brain controls a determinant of ion

flow in glutamate-gated channels. Cell. 2001; 67: 11-9.

28) Rueter SM, Dawson TR, Emeson RB. Regulation of alternative splicing by RNA editing. Nature. 1999; 399: 75-80.

29) Koh DS, Burnashev N, Jonas P. Block of native Ca^{2+}-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. J Physiol. 1995; 486: 305-12.

30) Jia Z, Agopyan N, Miu P, et al. Enhanced LTP in mice deficient in the AMPA receptor GluR2. Neuron. 1996; 17: 945-56.

31) Brusa R, Zimmermann F, Koh DS, et al. Early-onset epilepsy and postnatal lethality associated with an editing-deficient Glu R-B allele in mice. Science. 1995; 270: 1677-80.

32) Greger IH, Khatri L, Kong X, et al. AMPA receptor tetramerization is mediated by Q/R editing. Neuron. 2003; 40(4): 763-74.

33) Greger IH, Khatri L, Ziff EB. RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. Neuron. 2002; 34(5): 759-72.

34) Mahajan SS, Ziff EB. Novel toxicity of the unedited GluR2 AMPA receptor subunit dependent on surface trafficking and increased Ca^{2+}-permeability. Mol Cell Neurosci. 2007; 35: 470-81.

35) Kawahara Y, Kwak S, Sun H, et al. Human spinal motoneurons express low relative abundance of GluR2 mRNA: an implication for excitotoxicity in ALS. J Neurochem. 2003; 85: 680-9.

36) Takuma H, Kwak S, Yoshizawa T, et al. 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: 806-15.

37) Aizawa H, Kimura T, Hashimoto K, et al. Basophilic cytoplasmic inclusions in a case of sporadic juvenile amyotrophic lateral sclerosis. J Neurol Sci. 2000; 176: 106-13.

38) 郭　伸, 日出山拓人, 西本祥仁, 他. 孤発性ALSの脊髄前角におけるRNA編集異常と病型. 厚生労働科学研究費補助金難治性疾患克服研究事業神経変性疾患に関する調査研究班報告書. 2007. p. 64-5.

39) 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: 11-4.

40) Van Damme P, Braeken G, Callewaert G, et al. GluR2 deficiency accelerates motor neuron degeneration in a mouse model of amyotrophic lateral sclerosis. J Neuropathol Exp Neurol. 2005; 64: 605-12.

41) Kuner R, Groom AJ, Bresink I, et al. Late-onset motoneuron disease caused by a functionally modeified AMPA receptor subunit. Proc Natl Acad Sci U S A. 2005; 102: 5826-31.

42) 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. 22004; 13: 2183-96.

43) Spalloni A, Albo F, Ferrari F, et al. Cu/Zn-superoxide dismutase(GLY93-->ALA) mutation alters AMPA receptor subunit expression and function and potentiates kainite-mediated toxicity in motor neurons in culture. Neurobiol Dis. 2004; 15: 340-50.

44) Tortarolo M, Grignaschi G, Calvaresi N, et al. Glutamate AMPA receptors change in motor neurons of SOD1G93A transgenic mice and their inhibition by a noncompetitive antagonist ameliorates the progression of amyotrophic lateral sclerosis-like disease. J Neurosci Res. 2006; 83: 134-46.

45) Sun H, Kawahara Y, Ito K, et al. 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: 782-91.

46) Mackenzie IR, Bigio EH, Ince PG, et al. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. Ann Neurol. 2007; 61: 427-34.

47) 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. 2007; 113: 535-42.

48) Levine MS, Klapstein GJ, Koppel A, et al. Enhanced sensitivity of N-methyl-D-aspartate receptor activation in transgenic and knockin mouse models of Huntington's disease. J Neurosci Res. 1999; 58: 515-32.
49) Morton AJ, Leavens W. Mice transgenic for the human Huntington's disasese mutation have reduced sensitivity to kainic acid toxicity. Brain Res Bull. 2006; 52: 51-9.
50) Snider BJ, Moss JL, Revilla FJ, et al. Neocortical neurons cultured from mice with expanded CAG repeats in the huntingtin gene: unaltered vulnerability to excitotoxins and other insults. Neuroscience. 2003; 120: 617-25.
51) Zeron MM, Hansson O, Chen N, et al. Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease. Neuron. 2002; 33: 849-60.
52) Kwak S, Weiss JH. Calcium-permeable AMPA channel in neurodegenerative disease and ischemia. Curr Opin Neurobiol. 2006; 16: 281-7.
53) Sun H, Kawahara Y, Ito K, et al. Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ. Neurosci Res. 2005; 52: 228-34.
54) Higuchi M, Maas S, Single FN, et al. Point mutation in an AMPA recepotor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature. 2000; 406: 78-81.
55) Kawahara Y, Ito K, Sun H, et al. Regulation of glutamate receptor RNA editing and ADAR mRNA expression in developing human normal and Down's syndrome brains. Dev Brain Res. 2004; 148: 151-5.
56) Kawahara Y, Ito K, Sun H, et al. Low editing efficiency of GluR2 mRNA is associated with a low relative abundant of ADAR2 mRNA in white matter of normal human brain. Eur J Neurosci. 2003; 18: 23-33.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Neuroscience Research 54 (2006) 11–14
Neuroscience Research
www.elsevier.com/locate/neures

Underediting of GluR2 mRNA, a neuronal death inducing molecular change in sporadic ALS, does not occur in motor neurons in ALS1 or SBMA

Yukio Kawahara [a,1], Hui Sun [a], Kyoko Ito [a], Takuto Hideyama [a], Masashi Aoki [b], Gen Sobue [c], Shoji Tsuji [a], Shin Kwak [a,*]

[a] *Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *Department of Neurology, Tohoku University Graduate School of Medicine, Sendai, Japan*
[c] *Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

Received 7 July 2005; accepted 13 September 2005
Available online 12 October 2005

Abstract

Deficient RNA editing of the AMPA receptor subunit GluR2 at the Q/R site is a primary cause of neuronal death and recently has been reported to be a tightly linked etiological cause of motor neuron death in sporadic amyotrophic lateral sclerosis (ALS). We quantified the RNA editing efficiency of the GluR2 Q/R site in single motor neurons of rats transgenic for mutant human Cu/Zn-superoxide dismutase (SOD1) as well as patients with spinal and bulbar muscular atrophy (SBMA), and found that GluR2 mRNA was completely edited in all the motor neurons examined. It seems likely that the death cascade is different among the dying motor neurons in sporadic ALS, familial ALS with mutant SOD1 and SBMA.

Keywords: ALS; SOD1; Spinal and bulbar muscular atrophy; Motor neuron; RNA editing; GluR2; AMPA receptor; Neuronal death

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with selective loss of both upper and lower motor neurons, and familial cases are rare. The etiology of sporadic ALS remains elusive but recently deficient RNA editing of AMPA receptor subunit GluR2 at the Q/R site is reported in motor neurons in ALS that occurs in a disease-specific and motor neuron-selective manner (Kawahara et al., 2004; Kwak and Kawahara, 2005). Moreover, underediting of the GluR2 Q/R site greatly increases the Ca^{2+} permeability of AMPA receptors (Hume et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992), which may cause neuronal death due to increased Ca^{2+} influx through the receptor channel, hence mice with RNA editing deficiencies at the GluR2 Q/R site die young (Brusa et al., 1995) and mice transgenic for an artificial Ca^{2+}-permeable GluR2 develop motor neuron disease 12 months after birth (Kuner et al., 2005). Such evidence lends strong support to the close relevance of deficient RNA editing of the GluR2 at the Q/R site to death of motor neurons in sporadic ALS. However, although we and other researchers have demonstrated that dying neurons in several neurodegenerative diseases exhibit edited GluR2 (Kwak and Kawahara, 2005), it has not yet been demonstrated whether the underediting of GluR2 occurs in dying motor neurons in motor neuron diseases other than ALS. Such investigation is of particular importance since it will help clarify whether the molecular mechanism of motor neurons death is common among various subtypes of motor neurons.

ALS associated with the SOD1 mutation (ALS1) is the most frequent familial ALS (Rosen et al., 1993), and mutated human SOD1 transgenic animals have been studied extensively as a disease model of ALS1, yet the etiology of neuronal death in the animals has not been elucidated. Another example of non-ALS motor neuron disease is spinal and bulbar muscular atrophy (SBMA), which predominantly affects lower motor neurons with a relatively slow clinical course. Since the CAG

* Corresponding author. Tel.: +81 3 5800 8672; fax: +81 3 5800 6548.
E-mail address: kwak-tky@umin.ac.jp (S. Kwak).
[1] Present address: The Wistar Institute, Philadelphia, PA, USA.

0168-0102/$ – see front matter
doi:10.1016/j.neures.2005.09.006

Table 1
RNA editing efficiency of single motor neurons in SBMA

Case	Age at death (year)	Sex	No. of CAG repeats[a]	Postmortem delay (h)	GluR2(+) MN[b]	MN with 100% editing efficiency (% of GluR2(+) MN)
SBMA, case 1	71	M	48	2.5	12	12 (100)
SBMA, case 2	78	M	42	2.5	16	16 (100)
SBMA, case 3	60	M	44	1	16	16 (100)

[a] Number of CAG repeats in the androgen receptor gene.
[b] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

repeat expansion in the androgen receptor gene has been demonstrated in SBMA (La Spada et al., 1991), and pharmacological castration is therapeutically effective in animal models (Katsuno et al., 2002, 2003), the death cascade responsible for SBMA is likely different from sporadic ALS. In this paper, an investigation is carried out into whether or not the dying mechanism underlying sporadic ALS is the same as ALS1 and SBMA by determining the editing status of the GluR2 Q/R site in single motor neurons.

2. Materials and methods

The animals used in this study were $SOD1^{G93A}$ and $SOD1^{H46R}$ transgenic male rats (Nagai et al., 2001) ($n = 3$ each) that had exhibited progressive neuromuscular weakness with their littermates as the control ($n = 3$ each) (Table 2). The first sign of disease in these rats was weakness of their hindlimbs, mostly exhibited by the dragging of one limb. Onset of motor neuron disease was scored as the first observation of abnormal gait or evidence of limb weakness. The mean age of onset of clinical weakness for the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines was 122.9 ± 14.1 and 144.7 ± 6.4 days, respectively. As the disease progressed, the rats exhibited marked muscle wasting in their hindlimbs, and then in the forelimbs. The mean duration after the clinical expression of the disease in the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines was 8.3 ± 0.7 and 24.2 ± 2.9 days, respectively (Nagai et al., 2001). The rats were killed 3 days and 2 weeks after the onset for the $SOD1^{G93A}$ and $SOD1^{H46R}$ lines, respectively, and we examined their fifth lumbar cord. Animals were handled according to Institutional Animal Care and Use Committee approved protocols that are in line with the Guideline for Animal Care and Use by the National Institute of Health. Spinal cords were isolated after deep pentobarbiturate anesthesia. In addition, spinal cords were obtained at autopsy from three genetically confirmed patients with SBMA (Table 1). Written informed consent was obtained from all subjects prior to death or from their relatives, and the Ethics Committees of Graduate School of Medicine, the University of Nagoya and the University of Tokyo approved the experimental procedures used. Spinal cords were rapidly frozen on dry ice and maintained at −80 °C until use.

Table 2
RNA editing efficiency of single motor neurons in mutated human SOD1 transgenic rats

Case (n)	GluR2(+) MN[a]	MN with 100% editing efficiency (% of GluR2(+) MN)
$SOD1^{G93A}$-1	13	13 (100)
$SOD1^{G93A}$-2	21	21 (100)
$SOD1^{G93A}$-3	21	21 (100)
$SOD1^{H46R}$-1	19	19 (100)
$SOD1^{H46R}$-2	23	23 (100)
$SOD1^{H46R}$-3	20	20 (100)
$SOD1^{G93A}$, littermates (3)	22	22 (100)
$SOD1^{H46R}$, littermates (3)	20	20 (100)

[a] Motor neurons in which GluR2 RT-PCR amplifying product was detected.

Single motor neurons were isolated and collected into respective single test tubes that contained 200 μl of TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA, USA) using a laser microdissection system as previously described (Kawahara et al., 2003b, 2004) (LMD, Leica Microsystems Ltd., Germany) (Fig. 1a). After extracting total RNA from single neuron tissue, we analyzed the RNA editing efficiency at the GluR2 Q/R site by means of RT-PCR coupled with digestion of the PCR amplified products with a restriction enzyme Bbv-1 (New England BioLabs, Beverly, MA, USA) (Takuma et al., 1999; Kawahara et al., 2003a, 2004), and the editing efficiency was calculated by quantitatively analyzing the digests with a 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), as previously described (Kawahara et al., 2003a). Briefly, after gel purification using Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA), PCR products were quantified using a 2100 Bioanalyser. An aliquot (0.5 μg) was then incubated at 37 °C for 12 h with 10 × restriction buffer and 2 U of *Bbv*I in a total volume of 20 μl and inactivated at 65 °C for 30 min. The PCR products had one intrinsic *Bbv*I recognition sites, whereas the products originating from unedited GluR2 mRNA had an additional recognition site. Thus, restriction digestion of the PCR products originating from edited rat (278 bp) and human (182 bp) GluR2 mRNA should produce two bands (human GluR2 in parenthesis) at 219 (116) and 59 (66) bp, whereas those originating from unedited GluR2 mRNA should produce three bands at 140 (81), 79 (35), and 59 (66) bp. As the 59 (66) bp band would originate from both edited and unedited mRNA, but the 219 (116) bp band would originate from only edited mRNA, we quantified the molarity of the 219 (116) and 59 (66) bp bands using the 2100 Bioanalyser and calculated the editing efficiency as the ratio of the former to the latter for each sample.

The following primers were used for PCR for rat and human GluR2 (amplified product lengths are also indicated): for rat GluR2 (278 bp): rF (5′-AGCAGATTTAGCCCCTACGAG-3′) and rR (5′-CAGCACTTTCGATGGGAGACAC-3′). for human GluR2, the first PCR (187 bp): hG2F1 (5′-TCTGGTTTTCCTTGGGTGCC-3′) and hG2R1 (5′-AGATCCTCAGCACTTTCG-3′); for the nested PCR (182 bp): hG2F2 (5′-GGTTTTCCTTGGGTGCCTTTAT-3′) and hG2R2 (5′-ATCCTCAGCACTTTCGATGG-3′). We confirmed that these primer pairs were situated in two distinct exons with an intron between them and did not amplify products originating from other GluR subunits (data not shown). PCR amplification for rat GluR2 was initiated with a denaturation step that was carried out at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. PCR amplification for human GluR2 began with a 1 min denaturation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 64 °C for 30 s and extension at 68 °C for 60 s. Nested PCR was conducted on 2 μl of the first PCR product under the same conditions with the exception of the annealing temperature (66 °C).

3. Results

The number of motor neurons was severely decreased in the spinal cord of SBMA patients, and we analyzed 44 neurons dissected from three cases (12 from case 1, 16 from cases 2 and 3). Restriction digestion of the PCR products yielded only 116 and 66 bp fragments but no 81 or 35 bp fragments as seen in ALS motor neurons in all the SBMA motor neurons examined. Likewise, restriction digestion of the PCR products from motor

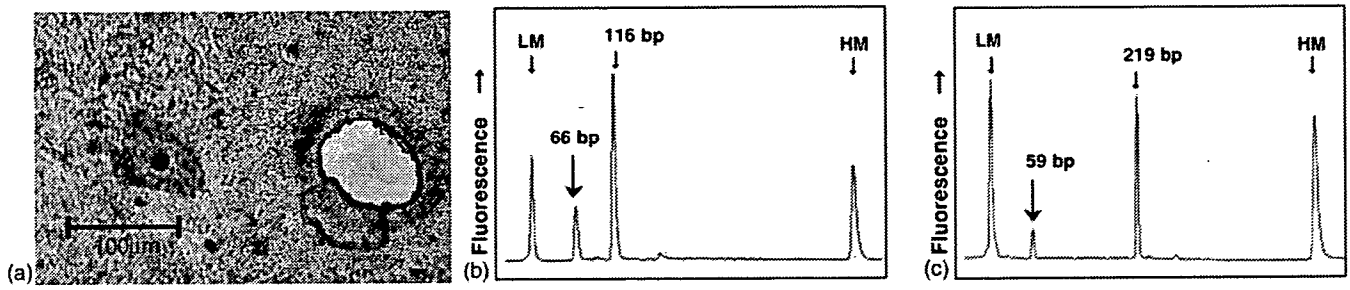


Fig. 1. (a) A single motor neuron from an SBMA patient before (left) and after (right) the dissection with a laser-microdissector. (b and c) An example of electropherogram by a 2100 Bioanalyser. Samples are the Bbv-1-digest of PCR product from tissues of a single motor neuron from an SBMA patient (b) and from a mutated human $SOD1^{G93A}$ transgenic mouse (c). LM: lower marker (15 bp), HM: higher marker (600 bp).

neurons of mutated human SOD1 transgenic rats yielded only 219 and 59 bp fragments (Fig. 1). Therefore, the values of RNA editing efficiency at the Q/R site of GluR2 were 100% in 44 motor neurons from three SBMA cases (Table 1), 55 single motor neurons from three $SOD1^{G93A}$ transgenic rats, 62 neurons from three $SOD1^{H46R}$ transgenic rats, as well as in 42 neurons from three littermate rats of each group (Table 2). The consistent finding that the GluR2 Q/R site is 100% edited in motor neurons of SBMA patients and transgenic rats for mutated human SOD1 is in marked contrast to the finding in ALS motor neurons that the editing efficiency widely varied among neurons ranging from 0% to 100% (Kawahara et al., 2004).

4. Discussion

Compared to the significant underediting reported for the GluR2 Q/R site in motor neurons of sporadic ALS (Kawahara et al., 2004), GluR2 mRNA in all the examined motor neurons of the mutated human SOD1 transgenic rats with two different mutation sites and SBMA patients was completely edited at the Q/R site. We have confirmed that postmortem delay hardly influenced the editing efficiency at the GluR2 Q/R site (Kawahara et al., 2003b), hence the significant difference in the postmortem delay between the SBMA patients in this study and ALS patients in the previous report (Kawahara et al., 2004) would not have affected these results. We examined the motor neurons in the spinal cord segment corresponding to the hindlimb of mutated human SOD1 transgenic rats after their hindlimbs had become weak, indicating that the motor neurons examined were already pathologically affected. Likewise, we found that only a small number of motor neurons remained in the spinal cord of SBMA patients. Thus our results indicate that GluR2 RNA editing was complete in the dying motor neurons in both the mutated human SOD1 transgenic rats and SBMA patients, implying that the neuronal death mechanism is not due to the underediting of GluR2 mRNA seen in sporadic ALS. Since the pathogenic mechanism underlying ALS1 is considered to be the same as in mutant human SOD1 transgenic animals, motor neurons in affected ALS1 patients would be expected to have only edited GluR2 mRNA. Indeed, an association study of the SOD1 gene in a considerable number of patients with sporadic ALS reported no significant association with mutations of the SOD1 gene (Jackson et al., 1997). Due to the lack of appropriate animal model for sporadic ALS, mutant human SOD1 transgenic animals have been used as a model for ALS in general, particularly in studies searching for therapeutically effective drugs. However, it should be kept in mind that mutated human SOD1 transgenic animals are merely a suggestive model for sporadic ALS and a gain of toxic function in mutated SOD1 kills motor neurons via mechanisms other than the demise of RNA editing. There are likely multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades.

Acknowledgements

This investigation was supported in part by grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan and grants from the Ministry of Health, Labor and Welfare of Japan (to SK), and a grant from Japan ALS Association (to YK).

References

Brusa, R., Zimmermann, F., Koh, D., Feldmeyer, D., Gass, P., Seeburg, P., Sprengel, R., 1995. Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. Science 270, 1677–1680.

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

Hume, R.I., Dingledine, R., Heinemann, S.F., 1991. Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253, 1028–1031.

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

Katsuno, M., Adachi, H., Doyu, M., Minamiyama, M., Sang, C., Kobayashi, Y., Inukai, A., Sobue, G., 2003. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. Nat. Med. 9, 768–773.

Katsuno, M., Adachi, H., Kume, A., Li, M., Nakagomi, Y., Niwa, H., Sang, C., Kobayashi, Y., Doyu, M., Sobue, G., 2002. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Neuron 35, 843–854.

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

Kawahara, Y., Ito, K., Sun, H., Kanazawa, I., Kwak, S., 2003a. 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. 18, 23–33.

Kawahara, Y., Kwak, S., Sun, H., Ito, K., Hashida, H., Aizawa, H., Jeong, S.-Y., Kanazawa, I., 2003b. Human spinal motoneurons express low relative abundance of GluR2 mRNA: an implication for excitotoxicity in ALS. J. Neurochem. 85, 680–689.

Kuner, R., Groom, A.J., Bresink, I., Kornau, H.C., Stefovska, V., Muller, G., Hartmann, B., Tschauner, K., Waibel, S., Ludolph, A.C., Ikonomidou, C., Seeburg, P.H., Turski, L., 2005. Late-onset motoneuron disease caused by a functionally modified AMPA receptor subunit. Proc. Natl. Acad. Sci. U.S.A. 102, 5826–5831.

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

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E., Fischbeck, K.H., 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. Nature 352, 77–79.

Nagai, M., Aoki, M., Miyoshi, I., Kato, M., Pasinelli, P., Kasai, N., Brown Jr., R.H., Itoyama, Y., 2001. Rats expressing human cytosolic copper–zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. J. Neurosci. 21, 9246–9254.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.

Takuma, H., Kwak, S., Yoshizawa, T., Kanazawa, I., 1999. 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. 46, 806–815.

Verdoorn, T., Burnashev, N., Monye, R.H., Seeburg, P., Sakmann, B., 1991. Structural determinants of ion flow through recombinant glutamate receptor channels. Science 252, 1715–1718.

ELSEVIER

Calcium-permeable AMPA channels in neurodegenerative disease and ischemia

Shin Kwak[1] and John H Weiss[2]

Compelling evidence supports contributions of glutamate receptor overactivation ('excitotoxicity') to neurodegeneration in both acute conditions, such as stroke, and chronic neurodegenerative conditions, such as amyotrophic lateral sclerosis. However, anti-excitotoxic therapeutic trials, which have generally targeted highly Ca^{2+} permeable NMDA-type glutamate channels, have to date failed to demonstrate impressive efficacy. Whereas most AMPA type glutamate channels are Ca^{2+} impermeable, an evolving body of evidence supports the contention that relatively unusual Ca^{2+} permeable AMPA channels might be crucial contributors to injury in these conditions. These channels are preferentially expressed in discrete neuronal subpopulations, and their numbers appear to be upregulated in amyotrophic lateral sclerosis and stroke. In addition, unlike NMDA channels, Ca^{2+} permeable AMPA channels are not blocked by Mg^{2+}, but are highly permeable to another potentially harmful endogenous cation, Zn^{2+}. The targeting of these channels might provide efficacious new avenues in the therapy of certain neurological diseases.

Addresses
[1] Department of Neurology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, 113-8655 Tokyo, Japan
[2] 2101 Gillespie Building, University of California, Irvine, CA 92697-4292, USA

Corresponding author: Weiss, John H (jweiss@uci.edu)

Current Opinion in Neurobiology 2006, 16:281–287

This review comes from a themed issue on
Signalling mechanisms
Edited by Erin M Schuman and Peter H Seeburg

Available online 15th May 2006

0959-4388/$ – see front matter

DOI 10.1016/j.conb.2006.05.004

Introduction

Excessive extracellular exposure to glutamate, an excitatory neurotransmitter, is harmful to neurons and contributes to neurodegeneration in certain diseases of the central nervous system. In amyotrophic lateral sclerosis (ALS), toxic elevations of glutamate appear to result from loss or dysfunction of astrocytic glutamate transporters. In ischemia, rapid glutamate release combined with deficiency in (or even reversal of) uptake causes extracellular glutamate accumulation.

Glutamate activates a number of types of postsynaptic ion channels. Most prominent among these are NMDA (N-methyl-D-aspartic acid)-type glutamate channels, which are highly Ca^{2+} permeable, and AMPA (1-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate channels, which mediate most rapid excitatory neurotransmission and are generally Ca^{2+} impermeable. However, some AMPA channels are Ca^{2+} permeable and emerging evidence supports the idea that these unusual channels, which are preferentially expressed on discrete populations of neurons, might be crucial contributors to injury in both ALS and ischemia.

It is also apparent that the number of Ca^{2+} permeable AMPA channels is subject to regulation both in response to physiological patterns of synaptic activity and in certain pathological states. Specifically, whereas relatively few Ca^{2+} permeable AMPA channels are normally present on hippocampal pyramidal neurons (HPNs), the number of these channels can increase sharply after ischemia. By contrast, the motor neurons (MNs), which selectively degenerate in ALS, normally do possess substantial numbers of Ca^{2+} permeable AMPA channels. However, recent evidence suggests that the number of these channels might further increase in ALS. In this review, we discuss recent evidence for roles of Ca^{2+} permeable AMPA channels in disease, with particular emphasis on intriguing clues to their roles in ALS and ischemia.

What are Ca^{2+}-permeable AMPA channels, and how are they regulated?

Functional AMPA receptors are homo- or hetero-oligomeric assemblies that are composed of various combinations of four possible subunits, GluR1, GluR2, GluR3 and GluR4. The Ca^{2+} conductance of AMPA receptors differs markedly according to whether the GluR2 subunit is present or not. AMPA receptors that contain at least one GluR2 subunit have low Ca^{2+} conductance, whereas those lacking a GluR2 subunit are Ca^{2+} permeable [1]. These properties of GluR2 are generated post-transcriptionally by RNA editing at the Q/R site in the putative second membrane domain (M2), during which a glutamine (Q) codon is replaced by an arginine (R) codon [1]. The presence of this positively charged residue, the arginine, in the pore of the channel impedes Ca^{2+} permeation (Figure 1). Analyses of the RNA from adult rat, mouse, and human brains have demonstrated that almost all GluR2 mRNA in neurons is edited, whereas in the GluR1, GluR3 and GluR4 subunits glutamine remains at this crucial position. AMPA receptors containing an unedited GluR2 (GluR2Q) have high Ca^{2+} permeability [1,2].

Glossary
Permeability transition pore: A large conductance channel through the mitochondrial membranes, persistent opening of which has been associated with mitochondrial disruption, release of the apoptotic mediator, cytochrome C, and cell death.
Ventral root avulsion: An injury causing disruption of the connection among spinal motor neurons, which send their axons out of the spinal cord through the ventral root, and the muscles that they innervate.

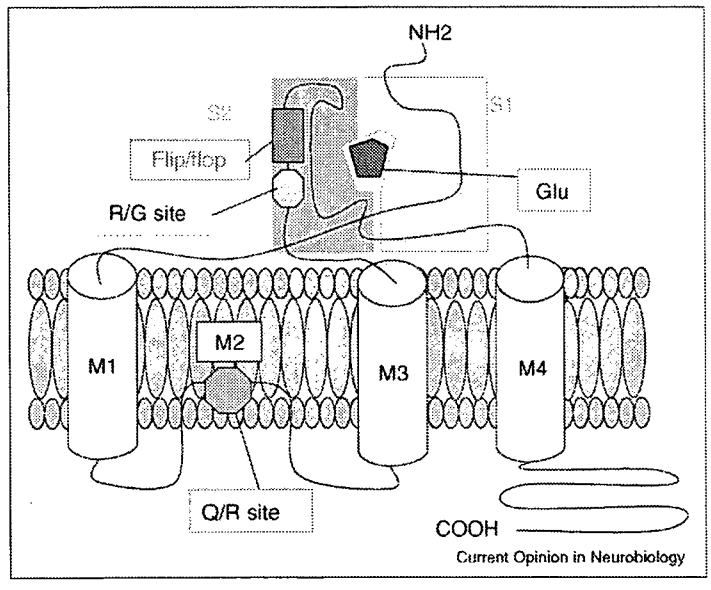
Under normal circumstances, most neurons have few Ca^{2+} permeable AMPA channels, reflecting the presence of edited GluR2 subunits and, therefore, arginine impeding Ca^{2+} entry in most of their AMPA channels. Furthermore, AMPA channels are not static, but undergo dynamic regulation through many mechanisms. Levels of Ca^{2+} permeable AMPA channels can be regulated by alterations in receptor trafficking, and a decrease in the number of these channels can occur in response to physiological activation [3], through mechanisms that appear to be dependent upon specific protein–protein interactions with GluR2 [4•,5•]. Indicating the importance of such regulation, insertion of AMPA channels into the synaptic membrane is sensitive to the editing state of the GluR2 Q/R site [6,7]. In contrast to the studies in which physiological activation results in a decrease in the number of postsynaptic Ca^{2+} permeable AMPA channels, recent studies have found that the presence of tumor necrosis factor-alpha (TNF-alpha), a cytokine, can result in membrane insertion of Ca^{2+} permeable AMPA channels in some neurons. This mechanism might promote neuronal injury in pathological conditions associated with elevations of this cytokine [8,9•,10•]. As discussed below, the number of Ca^{2+} permeable AMPA channels can also be regulated at the level of GluR2 mRNA expression, as is hypothesized to be the case in ischemia [11,12], or by defects in mRNA editing, as is hypothesized to be the case in ALS [13••,14•,15].

How might Ca^{2+} permeable AMPA channel activation injure neurons?

Although mechanisms of excitotoxic neuronal injury are complex and not completely understood, intracellular Ca^{2+} overload is an important trigger. With substantial intracellular Ca^{2+} loading, Ca^{2+} is taken up into mitochondria, and can cause generation of reactive oxygen species (ROS) or opening of the permeability transition pore (see glossary) and release of apoptotic mediators such as cytochrome C. With more modest intracellular Ca^{2+} accumulation, injury could be mediated by other mechanisms, including generation of nitric oxide (NO), with consequent activation of poly(ADP-ribose) polymerase (PARP) and release of mitochondrial apoptosis inducing factor (AIF) [16].

Another way in which Ca^{2+} permeable AMPA channels might mediate injury is by serving as entry routes for the divalent cation, Zn^{2+}, which is co-released with glutamate at certain excitatory synapses. Zn^{2+} accumulates in HPNs in ischemia and epilepsy, both conditions in which Zn^{2+} chelators are neuroprotective [17,18]. Whereas the Zn^{2+} accumulation is probably due to a combination of 'translocation' across the synapse and mobilization from

Figure 1



Structure of an AMPA receptor subunit (GluR2). The Q/R site is localized in the P-loop or putative second membrane domain (M2), which faces the channel pore of the AMPA receptor. Permeation of divalent cations is prevented when positively charged arginine (R) is placed in the Q/R site (through editing of the GluR2 mRNA), but can permeate when neutral glutamine (Q) is present. Reprinted with permission from Figure 1b in Nishimoto *et al.* [50].

intracellular pools [18], Ca^{2+} permeable AMPA channels are highly Zn^{2+} permeable, and might thus be the primary route for entry of synaptic Zn^{2+} [19–21]. Similar to Ca^{2+}, Zn^{2+} appears to induce injury through a number of mechanisms, including enzyme induction, ROS generation and PARP activation [22]. Intriguingly, however, in comparison to Ca^{2+}, Zn^{2+} is far more potent at inducing disruption of mitochondrial function, raising the possibility that mitochondrial effects contribute prominently to the degeneration resulting from strong intracellular Zn^{2+} accumulation [20,23–25].

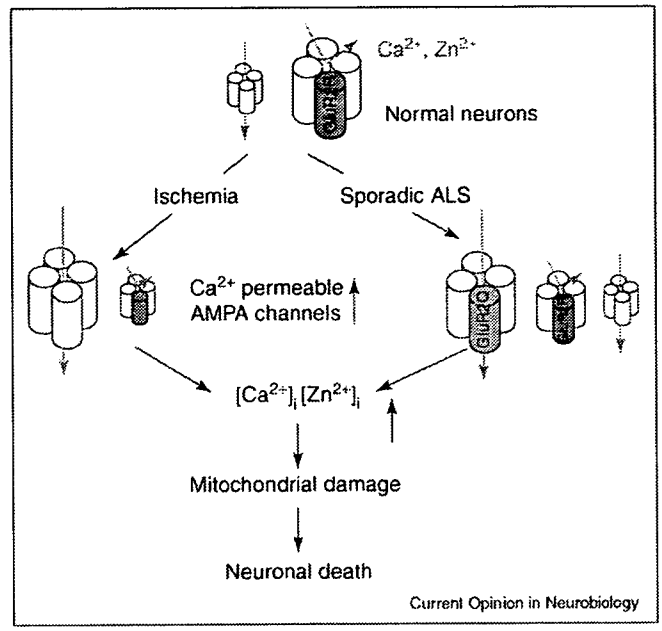
There are several reasons that Ca^{2+} permeable AMPA channels might be expected to play greater roles in neurodegeneration than NMDA channels. First, an increase in their number, as appears to happen in sporadic ALS and ischemia, would subject neurons to new metabolic burdens, possibly tipping the balance towards degeneration. Second, because NMDA channels are subject to voltage-dependent block by Mg^{2+} ions, they permit little Ca^{2+} entry in the absence of strong postsynaptic depolarization. Finally, the high Zn^{2+} permeability of Ca^{2+} permeable AMPA channels does not apply to NMDA channels, which are blocked by Zn^{2+}.

Role in amyotrophic lateral sclerosis

An excitotoxic model of ALS was supported by the observation that astrocytic glutamate uptake is deficient in the motor cortices and spinals cords of ALS patients [26]. Furthermore, the finding that MNs are selectively vulnerable to injury caused by AMPA/kainate receptor activation [27,28] suggested a crucial role for these receptors. This vulnerability is possibly caused by the fact that MNs possess substantial numbers of Ca^{2+} permeable AMPA channels [27,29,30], a finding consistent with the observation that MNs possess a lower relative abundance of GluR2 mRNA as compared with that in other neuronal subclasses both in humans [31] and in rats [32].

If Ca^{2+} permeable AMPA channels play a crucial role in excitotoxic MN injury, an increase in their number might initiate or accelerate the disease. Recent studies using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to compare GluR2 mRNA expression between ALS patients and controls found no differences in total GluR2 mRNA levels or the ratio of GluR2 mRNA to total AMPA receptor subunit mRNA [31]. However, these studies did find evidence for a reduction of GluR2 editing efficiency [13••,14•,15], which appeared to be selective for MNs [13••]. Furthermore, the editing defect appeared to be specific to ALS among several neurodegenerative diseases [14•], and to be specific to sporadic disease, as GluR2 mRNA was fully edited in G93A and H46R SOD1 transgenic rat models of familial ALS, and in humans with spinal and bulbar muscular atrophy (SBMA) [33•] (Figure 2).

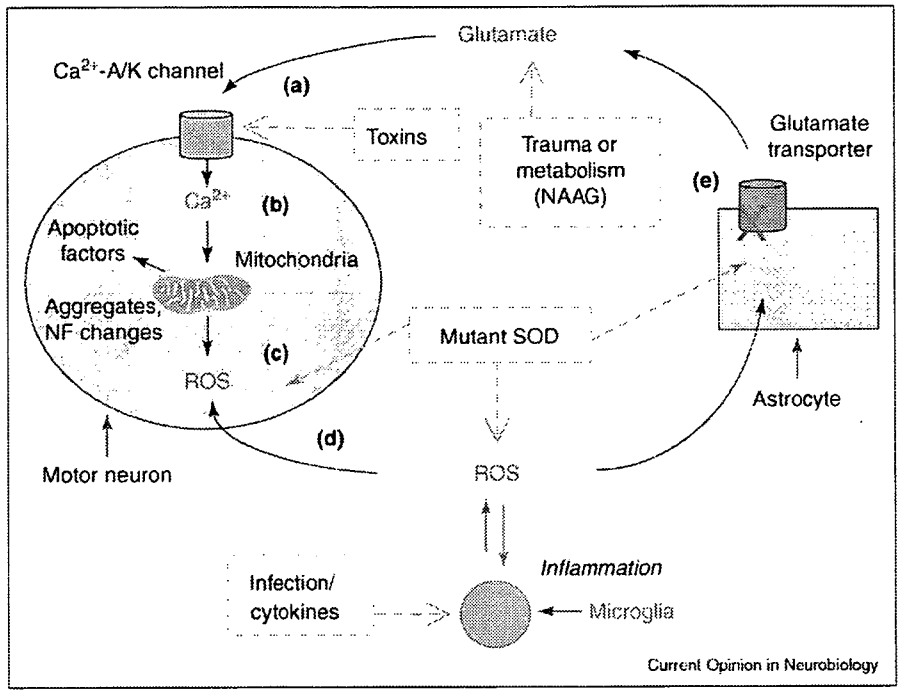
Figure 2



Ca^{2+} or Zn^{2+} influx through AMPA receptors is regulated by the GluR2 subunit. AMPA receptors with GluR2 that has been edited at the Q/R site are Ca^{2+}-impermeable, but those lacking GluR2 entirely or with unedited GluR2 are Ca^{2+}-permeable. Under basal conditions, hippocampal pyramidal neurons (HPNs) have relatively few Ca^{2+} permeable AMPA channels comprising AMPA receptors lacking GluR2, whereas motor neurons (MNs) possess a substantial number of these channels. After ischemia, a decrease in GluR2 mRNA expression in CA1 hippocampal pyramidal neurons results in increased numbers of Ca^{2+} permeable AMPA channels lacking GluR2, contributing to their delayed degeneration (Of note, a recent study suggests that loss of editing efficiency might also contribute to increased Ca^{2+} permeable AMPA channel expression after ischemia; see update). By contrast, MNs in sporadic ALS express considerable unedited GluR2 mRNA, probably resulting in an increased number of Ca^{2+} permeable AMPA channels containing unedited GluR2. In either case, an increase in the proportion of Ca^{2+} permeable AMPA channels enables increased Ca^{2+} and/or Zn^{2+} entry into the cytoplasm, which contributes to neuronal death partly through effects on mitochondria. Red, orange and white cylinders represent edited GluR2, unedited GluR2 and AMPA receptor subunits other than GluR2 (GluR1, 3 or 4), respectively.

Many recent studies support the theory that Ca^{2+} permeable AMPA channels have a crucial role in MN degeneration in diverse conditions. A recent study found ventral root avulsion (see glossary) to cause selective decreases in the GluR2 protein [34], probably contributing to the MN injury in that condition. A Ca^{2+} permeable AMPA channel blocker was found to be protective in a model of virus-induced MN degeneration [35•]. Transgenic animal studies have recently solidified the link between Ca^{2+} permeable AMPA channels and MN loss in SOD1-linked familial forms of ALS. Specifically, mice with modified GluR2 (GluR2-N), which results in production of AMPA channels with enhanced Ca^{2+} permeability, had late life MN degeneration [36••], and crossing either these mice or mice lacking GluR2 entirely with mice with the G93A

Figure 3



A feed-forward model of ALS pathogenesis. Present observations provide the basis for a feed-forward cycle leading to selective MN injury in ALS. **(a)** Elevations of extracellular glutamate induce **(b)** excessive Ca^{2+} entry into MNs (through Ca^{2+} permeable AMPA channels), where it is taken up by mitochondria, **(c)** with consequent ROS generation and, possibly, activation of apoptotic pathways, either of which would injure the neuron. **(d)** The ROS could pass across the MN plasma membrane and **(e)** disrupt astrocytic glutamate transporters, thereby causing further rises in extracellular glutamate. Such a cycle could, in principle, be triggered at different sites (e.g., via glutamate rises or oxidative stress), and thus might be compatible with a multiplicity of inciting mechanisms (suggested in dashed boxes) leading into a common self propagating disease pathway. Reprinted from [42•] with permission from Elsevier.

SOD1 model of ALS resulted in marked acceleration of the disease [37•]. Conversely, when mice with a decreased number of Ca^{2+} permeable AMPA channels in their MNs (via targeted GluR2 overexpression) were crossed with the G93A mice, the disease was significantly delayed [38•].

Mechanisms through which the presence of Ca^{2+} permeable AMPA channels contributes to excitotoxic MN injury are being elucidated. Although these channels enable rapid Ca^{2+} entry, MNs buffer cytosolic Ca^{2+} loads poorly [39], and consequently much of the Ca^{2+} is readily taken up into mitochondria, resulting in strong ROS generation [40,41]. Furthermore, *in vitro* studies indicated that ROS produced in MNs in response to Ca^{2+} permeable AMPA channel activation might induce oxidative dysfunction of glutamate transporters in surrounding astrocytes [42•]. This mechanism could play a role in the glutamate transport disruption seen in ALS, and provides the basis for a feed-forward cycle that could be integral to progression of the disease [42•] (Figure 3).

Role in ischemia

After transient global ischemia, HPNs, particularly in the CA1 subzone of the hippocampus, conspicuously degenerate, often with a delay of several days. Under basal conditions, HPNs have few Ca^{2+} permeable AMPA channels. However, recent studies suggest that limited numbers of these channels are present, and they appear to be mainly localized to dendritic branches remote from the soma, where they are difficult to detect electrophysiologically [43–45].

Observations that GluR2 mRNA is markedly and selectively downregulated in CA1 HPNs after ischemia have led to an hypothesis that consequent increases in the number of Ca^{2+} permeable AMPA channels contribute to the delayed neurodegeneration [12] (Figure 2). Studies in recent years have provided considerable support to this hypothesis. First, GluR2 protein levels are decreased and AMPA-mediated Ca^{2+} currents are increased after ischemia [11]. Also, increasing the number of Ca^{2+} permeable AMPA channels in CA1 resulted in an increased vulnerability of HPNs to ischemic injury [46], whereas elevating GluR2 levels appeared to be protective [47•]. Finally, some neuroprotection was observed upon addition of a Ca^{2+} permeable AMPA channel blocker many hours to days after the induction of ischemia [48••]. This suggests that new treatments targeting these channels could

provide therapeutic benefits in humans even when given well after the ischemic episode.

Intriguingly, recent studies suggest that Zn^{2+} has multiple roles in this delayed selective neurodegeneration. In an *in vitro* slice model of acute ischemia, addition of either an extracellular Zn^{2+} chelator or a Ca^{2+} permeable AMPA channel blocker decreased both Zn^{2+} accumulation and consequent neuronal injury [21]. In an *in vivo* animal model, addition of an extracellular Zn^{2+} chelator either before or several days (but not several hours) after ischemia afforded neuroprotection. The early application of the chelator attenuated the downregulation of GluR2, suggesting a role for Zn^{2+} in signaling the increase in Ca^{2+} permeable AMPA channels. Whereas, late application of chelator, after Ca^{2+} permeable AMPA channel numbers had already risen, attenuated the late rise in intracellular Zn^{2+} associated with injury, suggesting that Ca^{2+} permeable AMPA channel dependent intracellular Zn^{2+} accumulation contributes to the delayed injury [49•].

Conclusions

Recent findings, reviewed above, suggest that increasing the number of Ca^{2+} permeable AMPA channels might contribute crucially to neurodegeneration in sporadic ALS and ischemia. The increase in Ca^{2+} permeable AMPA channels in these conditions could be achieved through different mechanisms: deficiencies in GluR2 mRNA editing in sporadic ALS or decreased levels of GluR2 mRNA in ischemia (Figure 2). In addition, basal levels of Ca^{2+} permeable AMPA channels appear to contribute to familial ALS associated with SOD1 mutations. Ca^{2+} permeable AMPA channels are probably also involved in other conditions including epilepsy and Alzheimer's disease, in which decreases in levels of GluR2 have been reported.

There are presently no selective Ca^{2+} permeable AMPA channel antagonists available for human trials or even for systemic administration in animals. Yet, the fact that Ca^{2+} permeable AMPA channels only constitute a minority of AMPA channels on most neurons makes them particularly attractive targets for therapeutics, as it could be possible to block much of the current through these channels without causing the degree of functional impairment that would accompany a comparable level of NMDA or total AMPA channel blockade. Furthermore, because an increase in Ca^{2+} permeable AMPA channel number might be integral to their pathological roles in certain conditions, in addition to the development of pharmacological antagonists, strategies for reducing their numbers or preventing their upregulation might also provide useful avenues for therapy.

Update

In a recent study [51••], Peng *et al.* report that forebrain ischemia in adult rats selectively disrupts Q/R site editing and the expression of GluR2 subunit mRNA in vulnerable neurons. The authors provide further evidence that the editing defect contributes to the consequent neurodegeneration of CA1 HPNs. Thus, these data suggest that alterations of GluR2 editing might not be unique to ALS, and that this mechanism might also contribute to delayed neurodegeneration after transient ischemia.

Acknowledgements

This work was supported by National Institutes of Health grant NS36548 (JH Weiss), a grant from the Muscular Dystrophy Association (JH Weiss), and a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan 14017020, 15016030, 16015228 (S Kwak).

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. 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.

2. 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.

3. Liu SQ, Cull-Candy SG: **Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype.** *Nature* 2000, **405**:454-458.

4. • Gardner SM, Takamiya K, Xia J, Suh JG, Johnson R, Yu S, Huganir RL: **Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF.** *Neuron* 2005, **45**:903-915.

The authors demonstrate specific interactions between the GluR2 subunit of AMPA receptors and the cellular proteins involved in receptor trafficking. These interactions appear to underlie decreases in Ca^{2+} permeable AMPA channel numbers in response to physiological activation.

5. • Liu SJ, Cull-Candy SG: **Subunit interaction with PICK and GRIP controls Ca^{2+} permeability of AMPARs at cerebellar synapses.** *Nat Neurosci* 2005, **8**:768-775.

These authors (like those above) demonstrate specific interactions between the GluR2 subunit of AMPA receptors and the cellular proteins involved in receptor trafficking. These interactions appear to underlie decreases in Ca^{2+} permeable AMPA channel numbers in response to physiological activation.

6. Greger IH, Khatri L, Kong X, Ziff EB: **AMPA receptor tetramerization is mediated by Q/R editing.** *Neuron* 2003, **40**:763-774.

7. Greger IH, Khatri L, Ziff EB: **RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum.** *Neuron* 2002, **34**:759-772.

8. Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC: **Control of synaptic strength by glial TNFalpha.** *Science* 2002, **295**:2282-2285.

9. • Ogoshi F, Yin HZ, Kuppumbatti Y, Song B, Amindari S, Weiss JH: **Tumor necrosis-factor-alpha (TNF-alpha) induces rapid insertion of Ca^{2+}-permeable alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/kainate (Ca-A/K) channels in a subset of hippocampal pyramidal neurons.** *Exp Neurol* 2005, **193**:384-393.

The authors use Ca^{2+} imaging techniques to demonstrate membrane insertion of Ca^{2+} permeable AMPA channel in subpopulations of hippocampal pyramidal neurons in response to exposure to the solubule cytokine, TNF-alpha.

10. Stellwagen D, Beattie EC, Seo JY, Malenka RC: **Differential**
• **regulation of AMPA receptor and GABA receptor trafficking by tumor necrosis factor-alpha**. *J Neurosci* 2005, **25**:3219-3228.
The authors report that TNF-alpha induces preferential exocytosis of GluR2-lacking AMPA receptors in hippocampal neurons.

11. Gorter JA, Petrozzino JJ, Aronica EM, Rosenbaum DM, Opitz T, Bennett MV, Connor JA, Zukin RS: **Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca^{2+} influx in hippocampal CA1 neurons of gerbil**. *J Neurosci* 1997, **17**:6179-6188.

12. Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS: **The GluR2 (GluR-B) hypothesis: Ca^{2+}-permeable AMPA receptors in neurological disorders**. *Trends Neurosci* 1997, **20**:464-470.

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.
The authors demonstrate that in sporadic ALS, MNs, but not Purkinje cells, have abundant GluR2 mRNA that is unedited at the Q/R site. The specificity of this finding to MNs and the demonstrated ability of the activation of Ca^{2+} permeable AMPA channels to injure neurons suggest that this molecular change might be tightly linked to the etiology of MN death in sporadic ALS.

14. Kwak S, Kawahara Y: **Deficient RNA editing of GluR2 and**
• **neuronal death in amyotropic lateral sclerosis**. *J Mol Med* 2005, **83**:110-120.
The authors review the AMPA receptor-mediated neuronal death in ALS, and discuss mechanisms that might underlie the underediting of GluR2 mRNA in MNs.

15. 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.

16. Hong SJ, Dawson TM, Dawson VL: **Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling**. *Trends Pharmacol Sci* 2004, **25**:259-264.

17. Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW: **The role of zinc in selective neuronal death after transient global cerebral ischemia**. *Science* 1996, **272**:1013-1016.

18. Lee JY, Kim JH, Palmiter RD, Koh JY: **Zinc released from metallothionein-iii may contribute to hippocampal CA1 and thalamic neuronal death following acute brain injury**. *Exp Neurol* 2003, **184**:337-347.

19. Jia Y, Jeng JM, Sensi SL, Weiss JH: **Zn^{2+} currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones**. *J Physiol* 2002, **543**:35-48.

20. Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH: **Preferential Zn^{2+} influx through Ca^{2+}-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production**. *Proc Natl Acad Sci USA* 1999, **96**:2414-2419.

21. Yin HZ, Sensi SL, Ogoshi F, Weiss JH: **Blockade of Ca^{2+}-permeable AMPA/kainate channels decreases oxygen-glucose deprivation-induced Zn^{2+} accumulation and neuronal loss in hippocampal pyramidal neurons**. *J Neurosci* 2002, **22**:1273-1279.

22. Kim YH, Koh JY: **The role of NADPH oxidase and neuronal nitric oxide synthase in zinc-induced poly(ADP-ribose) polymerase activation and cell death in cortical culture**. *Exp Neurol* 2002, **177**:407-418.

23. Jiang D, Sullivan PG, Sensi SL, Steward O, Weiss JH: **Zn^{2+} induces permeability transition pore opening and release of pro-apoptotic peptides from neuronal mitochondria**. *J Biol Chem* 2001, **276**:47524-47529.

24. Sensi SL, Ton-That D, Sullivan PG, Jonas EA, Gee KR, Kaczmarek LK, Weiss JH: **Modulation of mitochondrial function by endogenous Zn^{2+} pools**. *Proc Natl Acad Sci USA* 2003, **100**:6157-6162.

25. Bossy-Wetzel E, Talantova MV, Lee WD, Scholzke MN, Harrop A, Mathews E, Gotz T, Han J, Ellisman MH, Perkins GA *et al.*: **Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K^+ channels**. *Neuron* 2004, **41**:351-365.

26. Rothstein JD, Martin LJ, Kuncl RW: **Decreased glutamate transporter by the brain and spinal cord in amyotrophic lateral sclerosis**. *N Engl J Med* 1992, **326**:1464-1468.

27. 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.

28. Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW: **Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity**. *Proc Natl Acad Sci USA* 1993, **90**:6591-6595.

29. Van Den Bosch L, Vandenberghe W, Klaassen H, Van Houtte E, Robberecht W: **Ca^{2+}-permeable AMPA receptors and selective vulnerability of motor neurons**. *J Neurol Sci* 2000, **180**:29-34.

30. Vandenberghe W, Robberecht W, Brorson JR: **AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability**. *J Neurosci* 2000, **20**:123-132.

31. Kawahara Y, Kwak S, Sun H, Ito K, Hashida H, Aizawa H, Jeong S-Y, Kanazawa I: **Human spinal motoneurons express low relative abundance of GluR2 mRNA: An implication for excitotoxicity in ALS**. *J Neurochem* 2003, **85**:680-689.

32. Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S: **Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ**. *Neurosci Res* 2005, **52**:228-234.

33. Kawahara Y, Sun H, Ito K, Hideyama T, Aoki M, Sobue G, Tsuji S,
• Kwak S: **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.
Although animals carrying mutated human SOD1 are the most widely used models for ALS, this paper reports that in these animals, and in humans with SBMA, the GluR2 editing defects do not occur. This suggests that differing molecular mechanisms underlie sporadic and familial forms of ALS.

34. Nagano I, Murakami T, Shiote M, Abe K, Itoyama Y: **Ventral root avulsion leads to downregulation of GluR2 subunit in spinal motoneurons in adult rats**. *Neuroscience* 2003, **117**:139-146.

35. Darman J, Backovic S, Dike S, Maragakis NJ, Krishnan C,
• Rothstein JD, Irani DN, Kerr DA: **Viral-induced spinal motor neuron death is non-cell-autonomous and involves glutamate excitotoxicity**. *J Neurosci* 2004, **24**:7566-7575.
The authors report that Ca^{2+} permeable AMPA channel blockers offer protection against MN death caused by a viral infection, supporting the contention that Ca^{2+} permeable AMPA channel activation contributes to MN degeneration in diverse conditions.

36. Kuner R, Groom AJ, Bresink I, Kornau HC, Stefovska V, Muller G,
•• Hartmann B, Tschauner K, Waibel S, Ludolph AC *et al.*: **Late-onset motoneuron disease caused by a functionally modified AMPA receptor subunit**. *Proc Natl Acad Sci USA* 2005, **102**:5826-5831.
In this study, mice transgenic for GluR-B(N), an artificial gene resulting in increased Ca^{2+} permeability of AMPA channels, developed slow onset of MN loss, indicating that increasing Ca^{2+} permeable AMPA channel numbers (as probably occurs from underediting of GluR2) does result in preferential MN injury. Additional presence of mutant SOD1 in these animals accelerated disease progression, indicating synergism between Ca^{2+} permeable AMPA channel activation and mutant SOD1 in mediating MN degeneration.

37. 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.
The authors report that deletion of the GluR2 subunit caused increased Ca^{2+} permeable AMPA channel number in MNs and accelerated MN loss in SOD1 mutant mice, again indicating synergistic effects of Ca^{2+} permeable AMPA channel activation and mutant SOD1.

38. Tateno M, Sadakata H, Tanaka M, Itohara S, Shin RM, Miura M,
• Masuda M, Aosaki T, Urushitani M, Misawa H *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.

Taking the opposite approach to the two papers above [36••,37•], these authors find that decreasing the number of Ca^{2+} permeable AMPA channels in MNs (by targeted GluR2 overexpression) delays the MN loss and prolongs survival in SOD1 mutant mice. This provides further evidence for a role of Ca^{2+} permeable AMPA channel activation in this familial form of ALS.

39. Lips MB, Keller BU: **Endogenous calcium buffering in motoneurones of the nucleus hypoglossus from mouse.** *J Physiol* 1998, **511**:105-117.

40. Carriedo SG, Sensi SL, Yin HZ, Weiss JH: **AMPA exposures induce mitochondrial Ca^{2+} overload and ROS generation in spinal motor neurons in vitro.** *J Neurosci* 2000, **20**:240-250.

41. Rao SD, Yin HZ, Weiss JH: **Disruption of glial glutamate transport by reactive oxygen species produced in motor neurons.** *J Neurosci* 2003, **23**:2627-2633.

42. • Rao SD, Weiss JH: **Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis.** *Trends Neurosci* 2004, **27**:17-23.

The authors consider the evidence that ROS produced in MNs might account for disruption of astrocytic glutamate transport, and discuss how this mechanism could underlie a feed-forward model of ALS progression and provide a basis for observations that MN loss in ALS is 'non-cell-autonomous'.

43. Lerma J, Morales M, Ibarz JM, Somohano F: **Rectification properties and Ca^{2+} permeability of glutamate receptor channels in hippocampal cells.** *Eur J Neurosci* 1994, **6**:1080-1088.

44. Ogoshi F, Weiss JH: **Heterogeneity of Ca^{2+}-permeable AMPA/kainate channel expression in hippocampal pyramidal neurons: fluorescence imaging and immunocytochemical assessment.** *J Neurosci* 2003, **23**:10521-10530.

45. Yin HZ, Sensi SL, Carriedo SG, Weiss JH: **Dendritic localization of Ca^{2+}-permeable AMPA/kainate channels in hippocampal pyramidal neurons.** *J Comp Neurol* 1999, **409**:250-260.

46. Anzai T, Tsuzuki K, Yamada N, Hayashi T, Iwakuma M, Inada K, Kameyama K, Hoka S, Saji M: **Overexpression of Ca^{2+}-permeable AMPA receptor promotes delayed cell death of hippocampal CA1 neurons following transient forebrain ischemia.** *Neurosci Res* 2003, **46**:41-51.

47. • Liu S, Lau L, Wei J, Zhu D, Zou S, Sun HS, Fu Y, Liu F, Lu Y: **Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia.** *Neuron* 2004, **43**:43-55.

These authors varied Ca^{2+} permeable AMPA channel levels in both directions, and found that decreasing their number (by overexpression of edited GluR2) attenuated ischemic injury in the CA1 subfield, whereas increasing Ca^{2+} permeable AMPA channels (by overexpression of unedited GluR2) resulted in new ischemic damage to granule neurons.

48. •• Noh KM, Yokota H, Mashiko T, Castillo PE, Zukin RS, Bennett MV: **Blockade of calcium-permeable AMPA receptors protects hippocampal neurons against global ischemia-induced death.** *Proc Natl Acad Sci USA* 2005, **102**:12230-12235.

The authors provide electrophysiological evidence for a substantial rise in the number of Ca^{2+} permeable AMPA channels on CA1 pyramidal neurons at 42 h after transient global ischemia. In addition, they find that addition of a Ca^{2+} permeable AMPA channel blocker between 9 and 40 h after the ischemia was partially protective and markedly reduced the rise in Zn^{2+} levels observed to proceed neuronal death.

49. • Calderone A, Jover T, Mashiko T, Noh KM, Tanaka H, Bennett MV, Zukin RS: **Late calcium EDTA rescues hippocampal CA1 neurons from global ischemia-induced death.** *J Neurosci* 2004, **24**:9903-9913.

These authors report that addition of the Zn^{2+} chelator, Ca- EDTA, can decrease CA1 neuronal degeneration in distinct ways depending upon the time of delivery. Early Ca-EDTA (before ischemia) prevented the GluR2 downregulation, cytochrome C release and other indices of mitochondrial disruption. By contrast, late Ca-EDTA (48–60 h after ischemia), when GluR2 is already downregulated, attenuated the late Zn^{2+} rises observed to precede cell death. The findings of this and the previous paper [48••] support the therapeutic efficacy of Ca^{2+} permeable AMPA channel blockade or Zn^{2+} chelation when delivered long after an ischemic insult.

50. Nishimoto Y, Hideyama T, Kawahar Y, Kwak S: **Reduction of RNA editing of an AMPA receptor subunit GluR2 and death of motor neurons in ALS (in Japanese).** *Clinical Neuroscience* 2006, **24**:222-225.

51. •• Peng PL, Zhong X, Tu W, Soundarapandian MM, Molner P, Zhu D, Lau L, Liu S, Liu F, Lu Y: **ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia.** *Neuron* 2006, **49**:719-733.

The authors use single cell PCR to show that forebrain ischemia in adult rats selectively disrupts Q/R site editing through downregulation of the editing enzyme adar2. They also provide evidence that this editing defect contributes to the delayed selective degeneration of CA1 hippocampal pyramidal neurons.

J Mol Med (2005) 83: 110–120
DOI 10.1007/s00109-004-0599-z

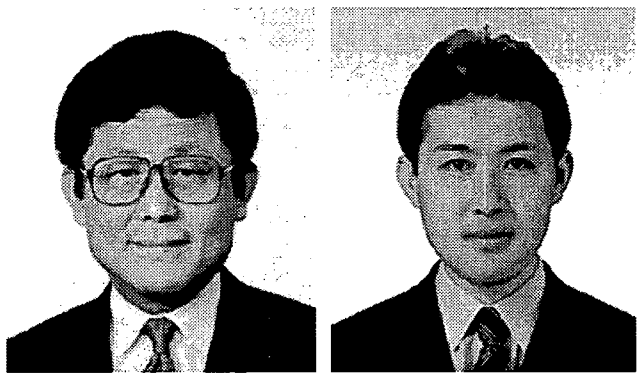
REVIEW

Shin Kwak · Yukio Kawahara

Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis

Received: 15 June 2004 / Accepted: 18 August 2004 / Published online: 29 December 2004

Abstract One plausible hypothesis for selective neuronal death in sporadic amyotropic lateral sclerosis (ALS) is excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, which are a subtype of ionotropic glutamate receptors. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether the GluR2 (or GluR-B) subunit is a component of the receptor. The properties of GluR2 are generated posttranscriptionally by RNA editing at the Q/R site in the putative second membrane domain (M2), during which the glutamine (Q) codon is substituted by an arginine (R) codon. AMPA receptors containing the unedited form of GluR2Q have high Ca^{2+} permeability in contrast to the low Ca^{2+} conductance of those containing the edited form of GluR2R. The role of Ca^{2+}-permeable AMPA receptors, particularly GluR2 Q/R site RNA editing status, in neuronal death has been clearly demonstrated both in mice deficient in editing at the GluR2 Q/R site and in mice transgenic for an artificial Ca^{2+}-permeable GluR2 subunit. We analyzed the expression level of mRNA of each AMPA receptor subunit in individual motor neurons, as well as the editing efficiency of GluR2 mRNA at the Q/R site in the single neuron level in control subjects and ALS cases. There was no significant difference as to the expression profile of AMPA receptor subunits or the proportion of GluR2 mRNA to total GluRs mRNA between normal subjects and ALS cases. By contrast, the editing efficiency varied greatly, from 0% to 100%, among the motor neurons of each individual with ALS, and was not complete in 44 of them (56%), whereas it remained 100% in normal controls. In addition, GluR2 editing efficiency was more than 99% in the cerebellar Purkinje cells of ALS, spinocerebellar degeneration and normal control groups. Thus, GluR2 underediting occurs in a disease specific and region selective manner. GluR2 modification by RNA editing is a biologically crucial event for neuronal survival, and its deficiency is a direct cause of neuronal death. Therefore, marked reduction of RNA editing in ALS motor neurons may be a direct cause of the selective motor neuron death seen in ALS. It is likely that the molecular mechanism underlying the deficiency in RNA editing is a reduction in the activity of ADAR2, a double- strand RNA specific deaminase. The restoration of this enzyme activity in ALS motor neurons may open the novel strategy for specific ALS therapy.



SHIN KWAK
is a neurologist and received his Ph.D. degree in medical science from the Faculty of Medicine, University of Tokyo, Japan. He is presently Associate Professor of the Department of Clinical Neurology, Graduate School of Medicine, University of Tokyo. His research interests include elucidation of mechanism underlying aberrant RNA editing in amyotropic lateral sclerosis motor neurons and development of novel specific therapy.

YUKIO KAWAHARA
after several training years as a neurologist, devoted himself to research on amyotropic lateral sclerosis etiology and received his Ph.D. degree in medical science this year from the Graduate School of Medicine, University of Tokyo. He is presently a research fellow at the Wistar Institute, Philadelphia, USA. His research interests include biological significance of RNA editing in normal and diseased human brains.

S. Kwak (✉) · Y. Kawahara
Department of Neurology, Graduate School of Medicine, University of Tokyo,
7-3-1 Hongo, Bunkyo-ku,
113-8655 Tokyo, Japan
e-mail: kwak-tky@umin.ac.jp
Tel.: +81-3-58008672
Fax: +81-3-58006548

Keywords ALS · amyotropic lateral sclerosis · AMPA receptor · Glutamate receptor · GluR2 · RNA editing · ADAR

Abbreviations *ADAR*: Adenosine deaminases acting on RNA · *ALS*: Amyotropic lateral sclerosis · *AMPA*: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate · *PCR*: Polymerase chain reaction

ALS: history

The most common motor neuron disease, amyotrophic lateral sclerosis (ALS), is characterized by a selective loss of upper and lower motor neurons that initiates in mid-life by a progressive paralysis with muscle wasting. ALS has a uniform worldwide prevalence (0.8–7.3 cases per 100,000 individuals), with risk of disease increasing in a age-dependent manner after the sixth decade of life, and only approximately 5–10% of all ALS cases are familial. Although three causal genes have been so far identified in individuals affected with familial ALS (SOD1, ALS2, senataxin) [1–4], the mechanism underlying motor neuron death or familial ALS pathology has not been elucidated. With regard to the non-hereditary form of ALS (sporadic ALS), which accounts for most cases of ALS, virtually no clues to the causal mechanism have been gleaned since the establishment of the disease entity by Jean-M. Charcot in 1874.

ALS etiology: the AMPA receptor-mediated neuronal death hypothesis

Several hypotheses have been proposed to explain the etiology of sporadic ALS, including genetic and/or non-genetic abnormalities of cytoskeletal and axonal transporting proteins [5, 6], vascular endothelial growth factor (VEGF) [7, 8], and viruses [9, 10], among others. Of these, one plausible hypothesis for selective neuronal death in sporadic ALS is excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, which are a subtype of ionotropic glutamate receptors. Because the pyramidal tract that projects to the motor neurons in the spinal cord uses glutamate as the excitatory neurotransmitter, motor neurons express abundant glutamate receptors and hence are vulnerable to exaggerated receptor activation by glutamate. The first act of the excitotoxicity story opened by selective loss of glutamate uptake due to loss of a glutamate transporter in the ALS motor cortex [11, 12], which was later proved to be not disease selective [13]. Including the neuronal death induced by glutamate transporter blockade, it has been repeatedly shown that motor neurons are differentially more vulnerable to AMPA receptor-mediated slow neuronal death than are other neuronal subsets in rat and mouse spinal cord cultures [14–16] and in the adult rat spinal cord [17, 18]. An increased influx of Ca^{2+} through activated AMPA receptor-coupled channels appears to play a key role in this type of neuronal death [19, 20].

Functional AMPA receptors are homo- or heterooligomeric 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 GluR2 subunit is a component of the receptor. AMPA receptors that contain at least one GluR2 subunit have low Ca^{2+} conductance, whereas those lacking a GluR2 subunit are Ca^{2+} permeable (Fig. 1A) [21–24]. These properties of GluR2 are generated posttranscriptionally by RNA editing at the Q/R site in the putative second membrane domain (M2; Fig. 1B), during which the glutamine (Q) codon is substituted by an arginine (R) codon [22, 23, 25]. Analyses of adult rat, mouse, and human brain RNA have demonstrated that almost all GluR2 mRNA in vivo is edited

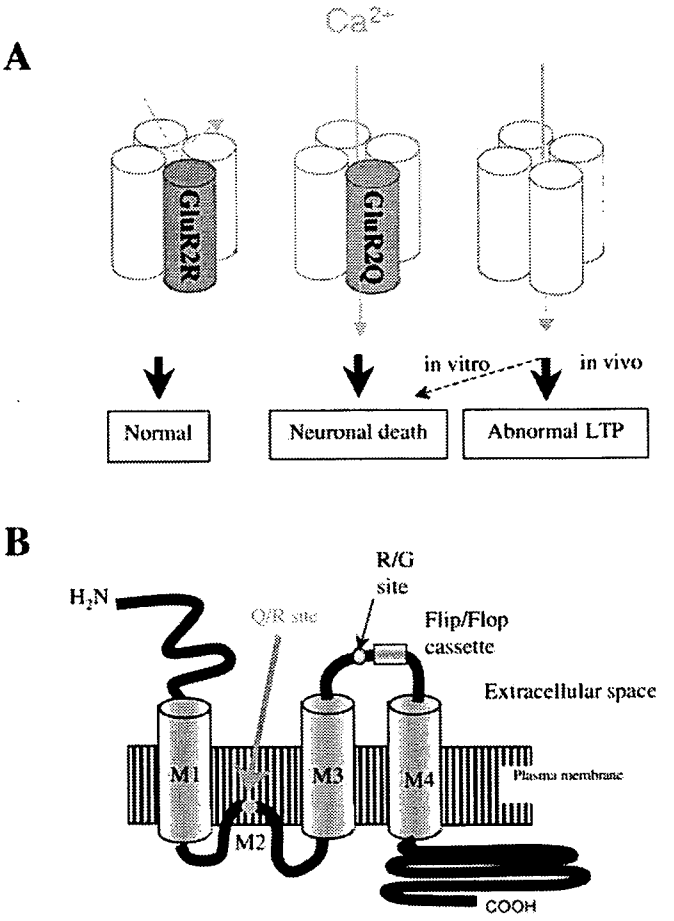


Fig. 1 Ca^{2+} permeability of AMPA receptors and the GluR2 subunit. **A** Functional AMPA receptors are homo- or heterooligomeric assemblies that comprise the four subunits GluR1, GluR2, GluR3, and GluR4 in various combinations. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether they contain the GluR2 subunit. AMPA receptors that contain at least one edited GluR2 subunit have low Ca^{2+} conductance (*left*), whereas those lacking a GluR2 subunit (*right*) and those containing unedited GluR2Q (*middle*) have high Ca^{2+} conductance. Neuronal death is induced when RNA editing at the GluR2 Q/R site is deficient. **B** Structure of GluR2: the Q/R site is located in the putative second membrane domain (*M2*)

(i.e., R is found at the Q/R site), whereas Q remains at this critical position in the GluR1, GluR3, and GluR4 subunits. AMPA receptors containing the unedited form of GluR2Q have high Ca^{2+} permeability in contrast to the low Ca^{2+} conductance of those containing the edited form of GluR2R (Fig. 1A) [26, 27].

The role of Ca^{2+}-permeable AMPA receptors in neuronal cell death has been demonstrated in animal models. Although low expression of GluR2 was found to influence AMPA receptor-mediated neuronal death in cultured hippocampal neurons from GluR2-null mice [28], low GluR2 expressed caused abnormal long-term potentiation (LTP) but not neuronal death in vivo [29]. By contrast, the effects of the GluR2 Q/R site RNA editing status on neuronal death have been clearly demonstrated both in mice deficient in editing at the GluR2 Q/R site [30] and in mice transgenic for an artificial Ca^{2+}-permeable GluR2 subunit [31]. Total Ca^{2+} influx depends on relative Ca^{2+} permeability, and on the number of open channels, as determined by the other factors including the proportion of flip/flop splice variants in AMPA receptor subunits and on the cellular density of AMPA receptors, although these factors rarely induce neuronal death.

Accordingly, two mechanisms have been proposed for the AMPA receptor-mediated neuronal death observed in ALS spinal motor neurons. The first is a selective reduction in GluR2 expression, which results in a decrease in the proportion of GluR2-containing, and therefore Ca^{2+}-impermeable, AMPA receptors owing to a low relative abundance of GluR2 among the four subunits. The second is a reduction in GluR2 RNA editing, which results in the number of GluR2Q-containing, Ca^{2+}-impermeable AMPA receptors increasing to a non-negligible amount.

AMPA receptor subunits in normal human CNS neurons

To our surprise, only inconsistent evidence had been available concerning the expression profile of AMPA receptor subunits in human brain. Therefore, we first quantified the expression level of each subunit in various neural subsets including spinal motor neurons. Notwithstanding studies reporting the expression of GluR2 [32, 33], some studies based on in situ hybridization or immunocytochemistry had claimed that GluR2 is not expressed in the spinal motor neurons of control human subjects [34, 35], and there was no evidence concerning whether or not GluR2 is present in ALS motor neurons. By means of a laser microdissector (Fig. 2) and real-time quantitative RT-PCR, we measured the relative abundance of GluR2 mRNA in situ in human spinal motor neurons and other neuronal tissues from control subjects. We found a stronger relative abundance of GluR2 mRNA, representing 77.8–95.5% of the total AMPA receptor subunits, as compared to other AMPA receptor subunit mRNAs throughout the neuronal subsets examined [36]. However, the motor neurons of control subjects expressed significantly lower levels of GluR2 (77.8±2.0%), as compared to other neuronal subsets (89.8±1.2% in Purkinje cells, 95.5±0.5% in cerebellar granule cells; Fig. 3) [36].

In rat spinal cords, the mRNA of all four AMPA receptor subunits has been detected in spinal motor neurons by in situ hybridization [37–40], and quantification of the relative abundance of GluR2 mRNA by single-cell RT-PCR has demonstrated a weaker predominance of GluR2 (from 2.1 to 63%) in cultured spinal motor neurons than what we found in human motor neurons [16, 41, 42]. The AMPA receptor subunit composition did not differ between motor neurons and dorsal horn neurons in immature rat dissociated spinal cord cultures [16], but significantly lower expression of GluR2 mRNA has been demonstrated in adult rat motor neurons than in dorsal horn neurons that are dissected with a laser microdissector (unpublished observation), which probably reflects age-dependent alterations of the AMPA receptor expression profile.

With regard to the other AMPA receptor subunits, the expression profiles differed among neuronal subsets, but were consistent between the same neuronal subsets analyzed from human and rat brains [36]. Low expression of GluR1 mRNA has been repeatedly shown in human and rat spinal motor neurons [36–38]. Rapid progress in the field of AMPA receptor trafficking has demonstrated the elaborate machinery that controls the activity-dependent trafficking of GluR1/GluR2 subunits differentially from the constitutive replacement of GluR2/GluR3 subunits [43, 44]. Thus, the different expression profiles of AMPA receptor subunits among neuronal subsets may reflect a subset-specific balance between activity-dependent and constitutive AMPA receptor regulatory mechanisms, rather than a species-specific difference.

Although the proportion of GluR2 varies relative to the other subunits, it is likely that GluR2 mRNA accounts for the major proportion of AMPA receptor subunits in neu-

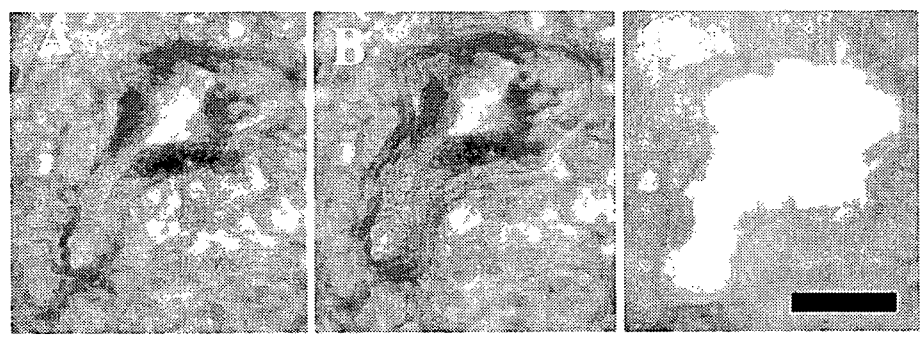


Fig. 2 Dissection of single motor neurons with a laser microdissector. **A** Before dissection; **B** demargination with a narrow laser beam; **C** after capturing the neuronal tissue. *Bar*= 40 μm

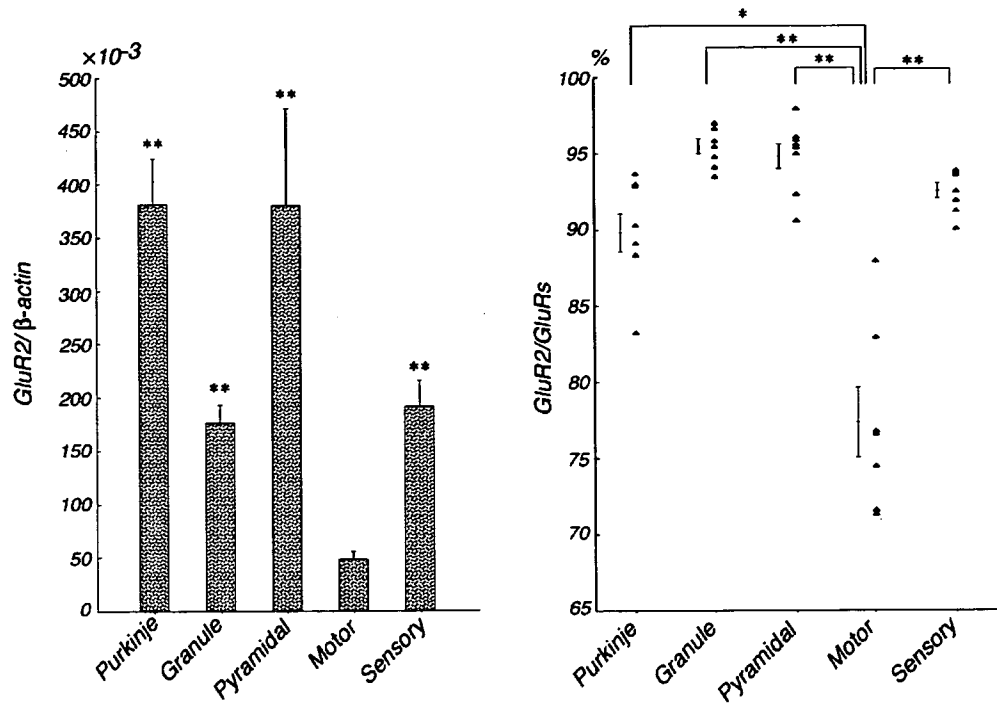


Fig. 3 Expression profile of AMPA subunit mRNA in single neuronal subsets of normal subjects. *Left*: The copy number of GluR2 mRNA has been normalized to the β-actin control (mean ±SEM; n=8). Samples significantly different from spinal motor neurons (*Motor*) are indicated (Mann-Whitney U test, $^{**}P<0.001$). *Right*: Each symbol represents the GluR2 mRNA copy number relative to the sum of the copy numbers of all GluR mRNAs (*GluRs*) in one subject. For each group, the mean ±SEM (n=8) is also displayed. The magnitude of GluR2 mRNA predominancy is significantly lower in spinal motor neurons (*Motor*) than in other neuronal subsets (Mann-Whitney U test, $^{*}P<0.01$, $^{**}P<0.001$). *Purkinje*, Purkinje cells; *Granule*, cerebellar granule cells; *Pyramidal*, cortical pyramidal neurons; *Sensory*, spinal dorsal horn neurons in substantia gelatinosa. (Modified from [36] with permission)

rons, including motor neurons of the spinal cords of humans as well as other mammals, and that the expression level of GluR2 mRNA is significantly lower in motor neurons than in other neuronal subsets in adult brains.

RNA editing

RNA editing has been defined as a posttranscriptional modification of the base sequence of mRNA and is recognized as a genetic mechanism for changing gene-specified codons and thus protein structure and function [45, 46]. In mammals, the main types of RNA editing are the base conversion of adenosine (A) to inosine (I) and that of cytidine (C) to uracil (U), which has been demonstrated to occur only in apolipoprotein B (ApoB) [47, 48] and neurofibromatosis type 1 (NF1) [49].

The conversion of A-to-I is most active in the central nervous system and has been found to occur predominantly in receptors and ion channels, including ionotropic glutamate receptor subunits [50–52], the serotonin -2C receptor $5HT_{2C}R$ [53], the voltage-dependent potassium channel Kv1.1 [54], and the RNA editing enzyme ADAR2 [55]. Among these, the biological effects of RNA editing have been most clearly demonstrated in ionotropic glutamate receptors, where editing positions have been identified in the subunits of AMPA receptors and kainate receptors, which, like AMPA receptors, are tetrameric assemblies comprising different combinations of the homologous subunits GluR5-GluR7, KA1, and KA2. Editing positions have been named after the amino acids that can occupy them, including the Q/R site in GluR2, GluR5, and GluR6 [50], the R/G site in GluR2, GluR3, and GluR4 [52], and the I/V and Y/C sites in GluR6 [51]. The change in amino acid residue caused by RNA editing, particularly at the Q/R site of GluR2, results in alterations in channel properties, including Ca^{2+} permeability [21, 22, 26, 27, 51, 56–59] and kinetic aspects of channel gating [52] in AMPA receptors.

AMPA receptors in ALS

As mentioned above, AMPA receptor-mediated neuronal death occurs after an increase in Ca^{2+} influx through AMPA receptor-coupled ion channels, and either a decrease in GluR2 expression or insufficient RNA editing at the GluR2 Q/R site is the causative molecular change. A decrease in GluR2 mRNA has been demonstrated after ischemia [60], but insufficient RNA editing had not been shown in any human neurological disease [61–63] or animal models under conditions inducing neuronal death after cerebral hypoxia [64] or kindling [65]. Owing to the lack of evidence on ALS motor neurons, we investi-

gated, both quantitatively and qualitatively, these molecular changes in AMPA receptors in motor neuron tissues of ALS patients, as compared to disease control and normal control subjects.

Quantitative analysis (RNA expression)

On the basis of the AMPA receptor mRNA expression profile in normal human neurons, we investigated whether the composition of AMPA receptor subunits is altered in ALS motor neurons. Quantitative analyses for AMPA receptor mRNA on both 100 motor neurons and single motor neurons showed that ALS motor neurons expressed almost the same amount of GluR2 mRNA relative to the β-actin baseline as control motor neurons (Fig. 4) [36]. Neither the ratio of β-actin mRNA-positive neurons to total dissected neurons (83.5% vs. 82.7%) nor the ratio of GluR2 mRNA-positive neurons to β-actin mRNA-positive neurons (57.9% vs. 53.7%) differed between the ALS and control groups, indicating that GluR2 mRNA expression is not altered in individual motor neurons of ALS [36]. Thus, selective reduction in GluR2 does not occur, and, therefore, an increase in the proportion of Ca^{2+}-permeable AMPA receptors owing to a reduced proportion of GluR2-containing AMPA receptors cannot be the mechanism underlying AMPA receptor-mediated neuronal death in ALS.

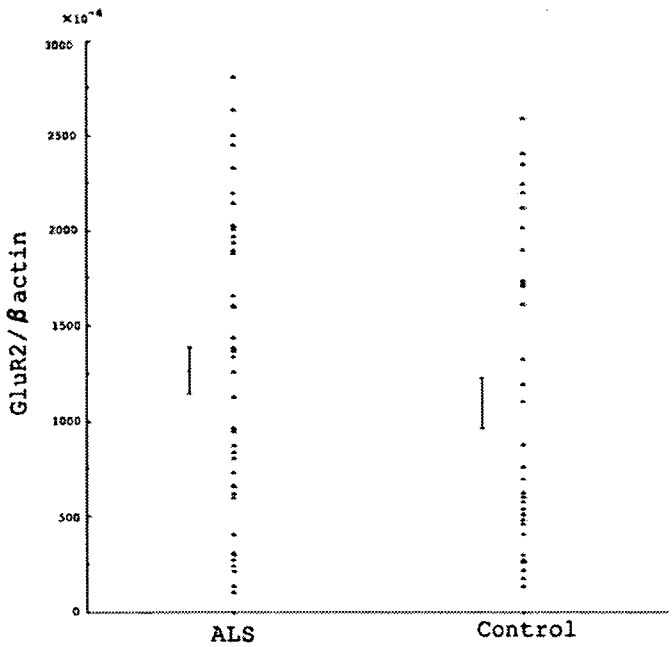


Fig. 4 Expression level of GluR2 mRNA in single motor neurons of ALS and control subjects. Each symbol represents the copy number of GluR2 mRNA normalized to the β-actin control in a single motor neuron. The expression levels in 44 motor neurons from three subjects with ALS and 36 motor neurons from three control subjects is shown. There is no significant difference between motor neurons from the ALS group and those from the control group (Mann-Whitney *U* test, $P>0.01$). For each group, the mean ±SEM is also displayed. (Adapted from [36] with permission.)

Qualitative analysis (RNA editing)

The alternative explanation for the AMPA receptor-mediated selective motor neuronal death in ALS is a deficiency in GluR2 Q/R site editing, which we have observed in the ventral gray matter of the ALS spinal cord [66]. In order to analyze neuron-selective tissue, we extracted RNA from single motor neurons isolated with a laser microdissector [36] from five individuals with ALS and five normal control subjects [67]. The editing efficiency was calculated by measuring differences in the digestion patterns of nested RT-PCR products of GluR2 mRNA obtained with the restriction enzyme *Bbv*I, whose cutting site depends on the occurrence of editing (Fig. 5A) [66–68]. The editing efficiency in GluR2 in cerebellar Purkinje cells was also quantified in individuals with ALS, dentatorubral-pallidoluysian atrophy (DRPLA), and multiple system atrophy (MSA) and compared with that in normal subjects.

The frequency of GluR2 mRNA positivity did not differ significantly between the ALS and the control groups (two-sample test for equality of proportions, $P>0.05$). The editing efficiency varied greatly, from 0% to 100%, among the motor neurons of each individual with ALS, and was not complete in 44 of them (56%); this was in marked contrast to the control motor neurons, of which all 76 examined showed 100% editing efficiency. The editing efficiency in Purkinje cells was virtually complete in the ALS, DRPLA, MSA, and normal groups (Fig. 5B) [67]. We confirmed that laser-captured neuronal tissue was not contaminated with tissues derived from glial cells or other cells, by PCR for microtubulus associate protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) on neural tissue and PCR for GluR2 on tissues from neuropil.

Albeit at a tissue level, GluR2 Q/R site editing has been reported to be preserved in the severely pathological brain areas of other neurodegenerative diseases including the striatum of Huntington disease, the neocortex and hippocampus of Alzheimer and Pick diseases, and the cerebellum of diseases of spinocerebellar degeneration [61–63, 69]. In addition, GluR2 editing was found to be virtually complete in Purkinje cells (Fig. 5B) [67] and the motor cortex [66] of ALS patients, indicating that the defect in GluR2 editing at the Q/R site is disease specific to ALS and site-selective to spinal motor neurons.

In accordance with our results, mice transgenic for an artificial Ca^{2+}-permeable GluR2 develop motor neuron disease late in life [31], indicating that motor neurons are specifically vulnerable to a deficiency in RNA editing. The proportion of the artificial Ca^{2+}-permeable GluR2 out of total GluR2 mRNA was only about 25% in that study, which raises questions about the type of molecular mechanism that could result in a small proportion of unedited GluR2Q altering the channel conductance of AMPA receptors to an extent sufficient to induce neuronal death. It has been reported that a GluR2Q subunit is more readily incorporated into functional AMPA receptors than is an edited GluR2R at the stage of tetramer (dimer of dimers) formation [70], as well as during receptor trafficking from