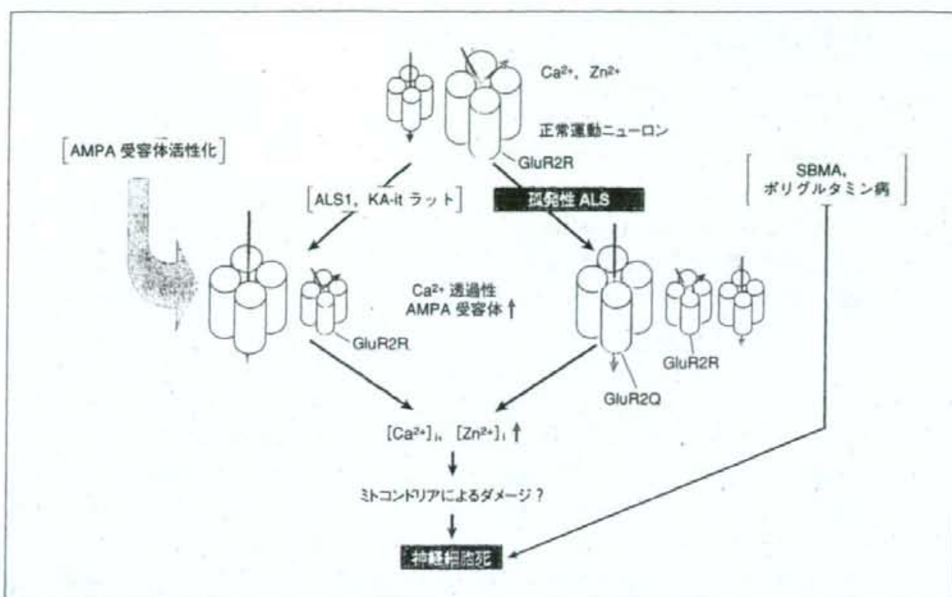

図9-3　運動ニューロン死のさまざまな分子機構
AMPA 受容体を介する神経細胞死を中心に正常の神経細胞ではほとんどの AMPA 受容体(AMPAR)は編集型 GluR2を含み Ca^{2+} 非透過性である．運動ニューロン(MN)などでは GluR2 を含まない Ca^{2+} 透過性の AMPA 受容体が，少数ながら存在していることが示されている．孤発性 ALS，変異 SOD1 関連家族性 ALS(ALS1)のいずれにも AMPA 受容体を介する細胞死のメカニズムが働いている証拠があるが，両者の分子機構は異なり，ALS1では GluR2 の割合の減少により編集型 GluR2 を含まない AMPA 受容体の割合が増えることにより（左），孤発性 ALS では未編集型 GluR2を含む AMPA 受容体が増えることにより(中)，Ca^{2+} 透過性の AMPA 受容体が増加し，細胞内 Ca^{2+} (Zn^{2+})濃度が上昇することが神経細胞死をひき起こす．ただし，後者が単独で神経細胞死をひき起こしうるのに対し，前者には変異 SOD1の細胞毒性などの因子がさらに加わる必要がある．これに対して，球脊髄性筋萎縮症（SBMA）の運動ニューロン死には，AMPA 受容体を介するメカニズムは働いていない．

る遅延傷害を阻止する[35]．

以上は GluR2 発現量低下による Ca^{2+} 透過性 AMPA チャネル増加のメカニズムを示している．一方，最近の研究で成体ラットの前頭葉脳虚血後に遅発性神経細胞死を生ずる海馬 CA1錐体細胞では，GluR2サブユニットの Q/R 部位編集と ADAR2 mRNA 量が選択的に減少していることが報告され，虚血後の遅発性神経細胞死においても GluR2 mRNA 編集低下による Ca^{2+} 透過性 AMPA チャネル増加のメカニズムが働いていることが示された[36]．

孤発性 ALS 運動ニューロンと虚血後の海馬 CA1 錐体細胞で同様の分子変化がみられることは，これら 2 種の神経細胞は未編集 GluR2 の増加による Ca^{2+} 透過性 AMPA チャネルを介した神経細胞死に，特に脆弱であることを示すのみならず，虚血に類似した環境因子が孤発性 ALS の運動ニューロン死をひき起こしうることをも示唆している．

9-6 ● 神経変性疾患と治療

上記のような最近の知見から Ca^{2+} 透過性 AMPA チャネルの増加は孤発性 ALS や脳虚血における神経変性に大きく寄与していることがわかる．Ca^{2+} 透過性 AMPA チャネルの増加のメカニズムは単一ではなく，GluR2 mRNA の編集異常，GluR2 の mRNA の減少（絶対量ないし，AMPA 受容体サブユニットに占める割合）がある（**図9-3**）．孤発性 ALS の運動ニューロン死は前者のメカニズムにより，前脳虚血後の海馬 CA1 錐体細胞の細胞死は両者のメカニズムが報告され，後者の例は SOD1 関連家族性 ALS の運動ニューロン，カイニン酸持続刺激による運動ニューロン死が挙げられる．後者のメカニズムによる Ca^{2+} 透過性 AMPA チャネルの増加が想定されている疾患としては，てんかんやアルツハイマー病なども挙げられるかもしれない．

このように Ca^{2+} 透過性 AMPA チャネルの増加が神経細胞死をひき起こすことが明らかである以上，このチャネルの活性を抑制する，このチャネルの増加を阻止するなどが疾患治療の戦略となりうる．現在のところ，ヒトへの臨床治験あるいは動物における全身投薬に利用できる Ca^{2+} 透過性 AMPA チャネルへの選択的なアンタゴニストはない．しかし，適切なアンタゴニストであれば，NMDA 受容体の興奮あるいは完全な AMPA チャネルを完全に遮断することなしに Ca^{2+} 透過性 AMPA チャネルを通る電流を遮断することは理論的に可能なので，増加した Ca^{2+} 透過性 AMPA チャネルを遮断することは，障害部位に対する標的治療になり，治療戦略としても魅力的である[1]．また，孤発性 ALS などにみられる Ca^{2+} 透過性 AMPA チャネル数増加は，RNA 編集の結果によりひき起こされるものであり，チャネル数の正常化による治療戦略も考えられる．

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

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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.
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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

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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-I (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 *BbvI* in a total volume of 20 μl and inactivated at 65 °C for 30 min. The PCR products had one intrinsic *BbvI* 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′-CAGCACTTTCGATGGAGACAC-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

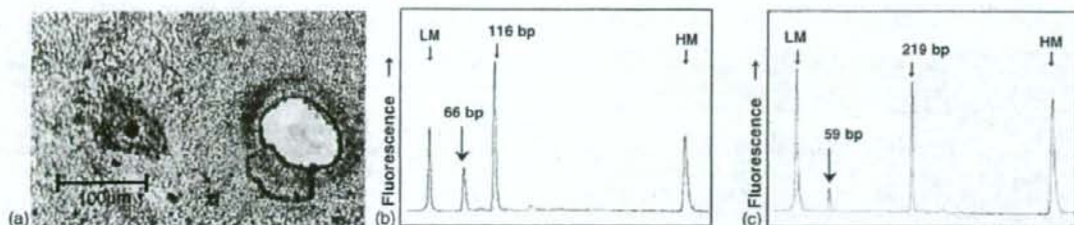

Fig. 1. (a) A single motor neuron from an SBMA patient before (left) and after (right) the dissection with a laser-microdissector. (b and c) An example of electropherogram by a 2100 Bioanalyser. Samples are the Bbv-1-digest of PCR product from tissues of a single motor neuron from an SBMA patient (b) 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).

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Journal of Neurochemistry, 2006, **98**, 782–791 doi:10.1111/j.1471-4159.2006.03903.x

Slow and selective death of spinal motor neurons *in vivo* by intrathecal infusion of kainic acid: implications for AMPA receptor-mediated excitotoxicity in ALS

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Abstract

Excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors has been proposed to play a major role in the selective death of motor neurons in sporadic amyotrophic lateral sclerosis (ALS), and motor neurons are more vulnerable to AMPA receptor-mediated excitotoxicity than are other neuronal subclasses. On the basis of the above evidence, we aimed to develop a rat model of ALS by the long-term activation of AMPA receptors through continuous infusion of kainic acid (KA), an AMPA receptor agonist, into the spinal subarachnoid space. These rats displayed a progressive motor-selective behavioral deficit with delayed loss of spinal motor neurons, mimicking the clinicopathological characteristics of ALS. These changes were significantly ameliorated by co-infusion with 6-nitro-7-sulfamobenso(f)quinoxaline-2,3-dione (NBQX), but not with D(–)-2-amino-5-phosphonovaleric acid (APV), and were exacerbated by co-infusion with cyclothiazide, indicative of an AMPA receptor-mediated mechanism. Among the four AMPA receptor subunits, expression of GluR3 mRNA was selectively up-regulated in motor neurons but not in dorsal horn neurons of the KA-infused rats. The up-regulation of GluR3 mRNA in this model may cause a molecular change that induces the selective vulnerability of motor neurons to KA by increasing the proportion of GluR2-lacking (i.e. calcium-permeable) AMPA receptors. This rat model may be useful in investigating ALS etiology.

Keywords: amyotrophic lateral sclerosis, AMPA receptor, excitotoxicity, GluR3, GluR2, RNA editing.

J. Neurochem. (2006) **98**, 782–791.

The most common motor neuron disease, amyotrophic lateral sclerosis (ALS), is a progressive neurodegenerative disease characterized by selective upper and lower motor neuron loss that is initiated in mid-life and leads to death as a result of respiratory muscular weakness. ALS has a uniform worldwide prevalence (five cases per 100 000 individuals), of which sporadic ALS accounts for more than 90% of all cases and only the remaining 5% of cases are familial (FALS) (Roland *et al.* 2005). Despite the fact that a good animal model is indispensable for investigating the etiology and for developing novel therapies for a disease, few appropriate animal models for sporadic ALS have been developed. Among the animal models tested, animal lines transgenic for the mutated human *Cu/Zn superoxide dismutase* (*SOD1*) gene (Gurney *et al.* 1994; Nagai *et al.* 2001; Howland *et al.* 2002) are regarded as a candidate disease model for all types of ALS, but the etiology of the FALS caused by the *SOD1* gene mutation is not necessarily the same as that of sporadic ALS. Indeed, the extent of neuropathological changes is not confined to motor neurons in these animals (Gurney *et al.* 1994), and although *SOD1* gene mutations have been found in a small population of patients with sporadic ALS, no significant association has been detected between sequence variants in the SOD1 locus and sporadic ALS

Received January 6, 2006; revised manuscript received March 15, 2006; accepted March 20, 2006.

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Abbreviations used: aCSF, artificial cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; APV, D(–)-2-amino-5-phosphonovaleric acid; CTZ, cyclothiazide; F, Fischer; FALS, familial ALS; KA, kainic acid; NBQX, 6-nitro-7-sulfamobenso(f)quinoxaline-2,3-dione; PBS, phosphate-buffered saline; SOD1, Cu/Zn superoxide dismutase; W, Wistar.

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susceptibility or phenotype (Jackson *et al.* 1997; Broom *et al.* 2004). Furthermore, the underediting of GluR2 mRNA at the Q/R site, which is a specific molecular change in sporadic ALS motor neurons (Kawahara *et al.* 2004), does not occur in degenerating motor neurons in rats transgenic for mutated human *SOD1* (Kawahara *et al.* 2006). Although *SOD1* transgenic animals have been used widely, these cannot be used as a model for sporadic ALS. On the other hand, a mouse line transgenic for Ca^{2+}-permeable artificial GluR-B(N) has been reported to develop motor neuron loss after 12 months of survival (Feldmeyer *et al.* 1999; Kuner *et al.* 2005), and therefore may be similar to sporadic ALS with respect to etiology, but this model has not been widely used.

Motor neurons in the spinal cord are differentially more vulnerable to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated excitotoxicity than are other neuronal subsets *in vitro* owing to an increased flux of Ca^{2+} through AMPA receptor channels (Carriedo *et al.* 1996). Ca^{2+} influx through AMPA receptors is regulated by either the presence or the absence of a GluR2 subunit: AMPA receptors containing GluR2 have a low Ca^{2+} influx, whereas those containing no GluR2 have a high Ca^{2+} influx (Hollmann *et al.* 1991; Verdoorn *et al.* 1991; Burnashev *et al.* 1992). However, when AMPA receptors contain a GluR2 subunit translated from unedited mRNA, their Ca^{2+} permeability remains high (Sommer *et al.* 1991). Therefore, both deficiency in GluR2 expression and underediting of GluR2 mRNA can induce AMPA receptor-mediated neuronal death. It is therefore interesting to investigate whether the AMPA receptor-mediated slow death of motor neurons occurs *in vivo* and what molecular changes occur in the AMPA receptor subunits. Here we induced the selective death of motor neurons in adult rats by a continuous infusion of kainic acid (KA) into the spinal subarachnoid space. Because slowly progressive and selective neuronal death is a hallmark of degenerative neurological diseases, such a model is useful to investigate the mechanism underlying the selective death of motor neurons in ALS.

Materials and methods

Operation

Each test compound was dissolved in artificial cerebrospinal fluid (aCSF; 122 mM NaCl, 3.1 mM KCl, 5 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4) with the pH adjusted to 7.2, and the resulting solution was used to fill an Alzet Model 2004 osmotic minipump (capacity 200 µL, pump speed 0.25 µL/ h; DURECT Corp., Cupertino CA, USA), which was incubated in sterile saline solution at 37°C overnight before operation after connection with a PE10 cannula (inner diamater, 0.25 mm; outer diamater, 0.55 mm; EICOM Corp., Tokyo, Japan). The operation was performed with slight modifications to the protocol described by Nakamura *et al.* (1994, 1997). In brief, male Wistar (W; body weight 180–250 g) and Fischer (F; body weight 110–160 g) rats (Japanese Oriental Yeast Co. Ltd, Shizuoko, Japan) were laminectomized at L5/6 under deep pentobarbiturate anesthesia, the free end of the PE10 cannula was inserted into the lumber spinal subarachnoid space, and the osmotic minipump was placed subcutaneously in the back.

Either W or F rats were infused with 3 mM KA (Sigma-Aldrich Corp., St Louis, MO, USA) continuously for 2 (KA-W2, KA-F2), 4 (KA-W4, KA-F4) or 8 (KA-W8) weeks. As controls, either W (aCSF-W) or F (aCSF-F) rats were infused with aCSF in the same manner for the same period of KA infusion (i.e. 2, 4 or 8 weeks). In some experiments, in order to investigate the neurotoxic mechanism, male F rats were infused intrathecally for either 2 or 4 weeks with one of the following combinations of glutamate receptor-acting drugs: 3 mM KA plus 3 mM 6-nitro-7-sulfamoyl-benzo(f)quinoxaline-2,3-dione (NBQX; TOCRIS Cookson Ltd, Bristol, UK) (KA/NBQX group); 3 mM KA plus 3 mM D(–)-2-amino-5-phosphonopentanoic acid (APV; TOCRIS NEURAMIN, Buckhurst Hill, Essex, UK) (KA/APV group); 1.5 mM cyclothiazide (CTZ; TOCRIS, Ellisville, MO, USA) (CTZ group); or 1.5 mM KA plus 1.5 mM CTZ (KA/CTZ group). In the KA/CTZ and CTZ groups, the osmotic minipump was replaced every week with one refilled with freshly prepared 1.5 mM KA plus 1.5 mM CTZ solution and 1.5 mM CTZ solution, respectively.

Animals were handled according to the protocols approved by the Institutional Animal Care and Use Committee in line with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Evaluation of movement behavior

Throughout the experimental period, the movement behavior of rats was measured each week, mainly by using a rat-specific rotarod (SN-445; Neuroscience Corp., Tokyo Japan), which constantly rotated at a speed of 16 rpm and automatically recorded the time that rats could stay on the rotarod during a 420-second period. The rotarod scores of rats were recorded on average in three separate trials. Paralysis was scored when the rat dragged one of its hindlimbs. Rats were also tested for whether they exhibited an escape response from the pain elicited by stimulation of the hindlimbs with tweezers, and whether they had urinary and/or fecal incontinence.

Spinal cord samples

The rats were anesthetized deeply using diethyl ether inhalant, after which their spinal cords were quickly removed and frozen in liquid nitrogen. The samples were stored at −80°C until use. The frozen spinal cord samples were obtained from KA-W2, KA-W4 and KA-W8 ($n = 5$ each); KA-F2 and KA-F4 ($n = 5$ each); KA/NBQX ($n = 5$); KA/APV ($n = 5$); KA/CTZ ($n = 5$); CTZ ($n = 5$); aCSF-W2, aCSF-W4 and aCSF-W8 ($n = 5$ each); and aCSF-F2 and aCSF-F4 ($n = 5$ each) rats.

For morphological analysis, rats were perfused transcardially with 3.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.6). The spinal cords of KA-W4 and KA-W8 ($n = 3$ each), KA-F2 and KA-F4 ($n = 3$ each), KA-NBQX ($n = 3$), KA/CTZ at 2 and 4 weeks ($n = 5$ each), CTZ ($n = 5$), aCSF-W4 and aCSF-W8 ($n = 3$ each), and aCSF-F2

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and aCSF-F4 (n = 5 each) rats were removed and postfixed in the same fixative at 4°C for 12 h.

Neuropathology

The lumber segments of spinal cords fixed in paraformaldehyde and glutaraldehyde were dehydrated overnight at 4°C with serial concentrations of sucrose (5%, 10%, 15%, 20%, 25% and 30%), and then rapidly frozen on dry ice. Thirty serial 20-µm-thick frozen sections were made with a cryostat (Model HM500 O; MICROM, Walldorf, Germany) and stained with either 0.1% Cresyl violet (pH 3.5) or hematoxylin and eosin. The number of neurons the diameters of which were greater than 20 µm and had identifiable nucleoli was counted in the ventral horns of the spinal cord under a light microscope, and their morphology was also observed.

To determine the morphological changes in the axons of motor neurons, L5 ventral roots of KA-W8 (n = 3), KA/CTZ (n = 3) and their respective control rats (n = 3 each) were postfixed with 2.0% paraformaldehyde and 2.0% glutaraldehyde in 0.1 M PBS, and mounted in resin. Transverse 1-µm-thick Epon-embedded sections of L5 ventral roots were made and stained with 0.1% toluidine blue and viewed under a light microscope.

Single-cell dissection

Single-cell isolation was carried out using an excimer laser microdissection system (Hamamatua Photonics Ltd, Shizuoka, Japan) as previously described (Hashida *et al.* 2001; Kawahara *et al.* 2003; Sun *et al.* 2005). In brief, 20-µm-thick frozen sections were attached to glass slides made of artificial quartz, fixed with 100% methanol for 60 s and then stained with 0.1% toluidine blue. Thirty spinal motor neurons (Motor) were dissected free and placed in test-tubes containing 200 µL of TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA). The substantia gelatinosa, ventral and dorsal funiculi of the spinal cord were dissected *en bloc* and placed in tubes containing 200 µL of TRIZOL reagent in a similar manner. All samples were stored at −20°C until use.

RNA extraction and reverse transcription

Total RNA was extracted from each tissue sample using TRIZOL reagent according to the manufacturer's instructions. Single-cell RNA extraction and reverse transcription procedures were carried out as previously described (Hashida *et al.* 2001; Kawahara *et al.* 2003; Sun *et al.* 2005). Reverse transcription was performed with either Ready-to-go You-Prime First-Strand beads (Amersham Biosciences Corp., Piscataway, NJ, USA) or 0.5 µM oligo(dT) primer for tissue samples, or with a Sensiscript RT Kit (QIAGEN GmbH, Hilden, Germany) using 0.5 mM oligo(dT) primer and 10 U of prime RNase inhibitor (Eppendorf AG, Hamburg, Germany) for the single-cell samples. The cDNAs were stored at −20°C until use.

Real-time quantitative PCR

The primer sets for the internal standard of GluR1–R4 and β-actin have been described elsewhere (Sun *et al.* 2005). Each PCR reaction was run in 50 µL of the reaction mixture containing each primer at 200 µM, 1 mM dNTP MIX, 5 µL of 10 × PCR buffer and 1 µL of Ampli Taq DNA polymerase (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ, USA). The PCR amplification conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. The PCR products were then purified with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA). The concentration of PCR products was determined with a spectrophotometer (Nano Drop™ ND-1000; Nano Drop Technologies Inc., Wilmington, DE, USA). We prepared serial dilutions with 10^8–10^6, 10^6–10^4, 10^4–10^3, 10^3–10^2 and 10^3–10^1 copies per 2 µL, which contained 100 ng of herring sperm DNA in Tris-EDTA buffer (pH 8.0).

The procedure was performed as described previously (Kawahara *et al.* 2003; Sun *et al.* 2005). In brief, quantitative PCR was performed with a LightCycler System (Roche Diagnostics, Mannheim, Germany). The PCR primers and probes for GluR1, GluR4 and β-actin comprised approximately 180–250 base pairs and matched sequences in the 3′ untranslated regions (UTR) in order to avoid the flip/flop and GluR4c alternative splicing sites. Moreover, phosphate groups were attached to the 3′ end of the LCRed640-containing probes to prevent probe extension. A set of standards and cDNA samples was amplified in duplicate in a reaction mixture (20 µL of total volume) containing 2 µL of 10 × FastStart Taq DNA polymerase, 4 mM $MgCl_2$, each primer at 0.5 mM, 0.2 µM fluorescein isothiocyanate (FITC)-containing probe and 0.4 µM LCRed640-containing probe. Herring sperm DNA solution was included as a negative control in each series of reactions. The reactions were initiated by the activation of FastStart Taq DNA polymerase at 95°C for 10 min, and amplification was achieved by running 45 cycles of denaturation at 95°C for 1 s, annealing at 58°C for 10 s, and extension at 72°C for 12 s.

Statistical methods

Differences between groups were evaluated by Mann–Whitney *U*-test. Significance was assumed at values of $p < 0.05$.

Results

Behavioral and morphological changes in the rats infused with KA

Because infusion of 1.5 mM KA did not cause any change in the rat for up to 8 weeks of continuous infusion, and because infusion of 4.5 mM KA induced death as a result of convulsion shortly after operation (data not shown), we adopted the concentration of 3.0 mM KA except in the experiments in which KA was co-infused with CTZ. As compared with the aCSF-infused control rats (aCSF-W/F), which maintained a score of 420 s, the rotarod scores of the KA-infused rats significantly decreased in a time-dependent manner after 2 weeks of KA administration in both the KA-W and the KA-F rat groups, reaching a seventh of the control score after 8 weeks (KA-W: week 0, 420 s, n = 15; week 2, 237.3 ± 40.4 s, mean ± SEM, n = 15; week 4, 205.4 ± 45.9 s, n = 10; week 8, 67.2 ± 19.0 s, n = 5) (KA-F: week 0, 420 s, n = 10; week 2, 179.7 ± 48.4 s, n = 10; week 4, 176.2 ± 40.1 s, n = 5) ($p < 0.001$; Fig. 1). Moreover, rats infused with KA developed complete paralysis of the hindlimbs by the end stage, whereas none of the rats showed either sensory deficits or urinary incontinence (data not shown).

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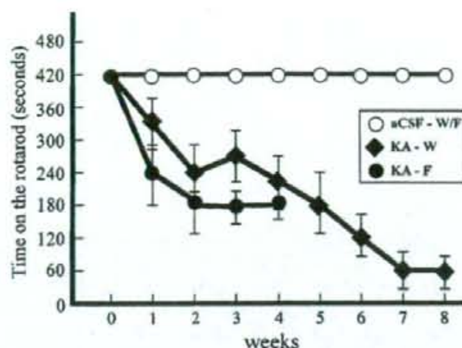

Fig. 1 Time-dependent motor deficit in kainic acid (KA)-infused rats. The rotarod scores of the KA-infused rats were significantly decreased in a time-dependent manner after 2 weeks of KA administration in both the Wistar and Fischer rat groups as compared with respective artificial cerebrospinal fluid (aCSF)-infused control rats ($p < 0.001$). The rotarod speed was 16 rpm.The values plotted on the graph represent the mean rotarod score; error bars indicate the SD. ◆, KA-infused Wistar rats; ●, KA-infused Fischer rats; ○, aCSF-infused Wistar/Fischer rats.

The motor neurons of the KA-F4 and the KA-W8 rats that displayed selective motor dysfunction exhibited morphological changes, including cytoplasmic microvacuolation, loss of Nissl substance and accumulation of glial cells around the degenerating neurons (Figs 2a and c), whereas neurons in the dorsal horn, including small neurons in the substantia gelatinosa of the same rats, appeared morphologically normal (Figs 2a and b). Axons in the fifth lumber ventral root of the KA-W8 rats were severely damaged and displayed a reduction in large myelinated fibers as compared with control rats (Figs 2d and e). Moreover, the number of large motor neurons was significantly reduced in the ventral horn of KA-F4 and KA-W8 rats (KA-F4, 4.8 ± 0.2; aCSF-F4, 6.2 ± 0.2; KA-W8, 3.2 ± 0.1; aCSF-W8, 6.0 ± 0.2; mean ± SEM; $n = 5$) ($p < 0.001$; Fig. 2f) but not in that of either KA-F2 or KA-W4 rats ($p > 0.1$; Fig. 2f). Taken together, these findings show that the KA-infused rats exhibited selective impairment in motor function, which is probably a result of the selective loss of motor neurons in the spinal cord.

Behavioral and morphological changes in the rats co-infused with KA and antagonists

Rats that were infused with KA and NBQX, a potent antagonist for AMPA and KA receptors, showed significantly higher rotarod scores after 3 weeks as compared with those infused with KA alone, whereas those infused with KA and APV, an NMDA receptor antagonist, did not differ from the KA-infused control rats (Fig. 3). By contrast, rats co-infused with CTZ, an AMPA receptor desensitization blocker, and the lower concentration of KA (1.5 mM KA + 1.5 mM CTZ) exhibited very low rotarod scores after 1 week, hence the co-infusion of CTZ and KA seemed to induce a more rapid decline of motor function as compared with the infusion of a higher concentration of KA alone (3 mM KA) (at week 1, $p < 0.05$; Fig. 3). Furthermore, rats that were infused with CTZ alone (1.5 mM CTZ) showed slightly, if any, and only transiently, lower rotarod scores as compared with aCSF-infused control rats (Fig. 3).

The morphometry of large neurons demonstrated that the decrease in the number of motor neurons was significantly less extensive in the KA/NBQX group than in the KA group after 4 weeks (KA, 4.8 ± 0.2; KA/NBQX, 5.6 ± 0.1; mean ± SEM; $n = 5$; $p < 0.001$), but not after 2 weeks (Fig. 4a). Although the concentration of KA in the KA/CTZ group was half of that in the KA-alone group, after 4 weeks the number of motor neurons in the KA/CTZ group tended to be lower than that in the KA group, and was significantly lower than that in the aCSF control and CTZ alone group (Fig. 4a). Moreover, motor neurons in the KA/CTZ group exhibited more severe morphological changes than did motor neurons in the KA-F4 (Fig. 4b) and KA-W8 groups (Fig. 2c), including numerous large cytoplasmic vacuoles with condensation of Nissl substance (Fig. 4c). After 4 weeks, the ventral roots of the KA/CTZ rats displayed marked axonal degeneration with loss of fibers as compared with the control rats (Figs 4d and e), which appeared rather more severe than the changes seen in the KA-W8 group (Fig. 2e).

Neuronal mRNA expression profile of AMPA receptor subunits

In motor neurons of the KA-infused groups (KA-W2, KA-W4, KA-W8, KA-F2 and KA-F4), the quantities of total AMPA receptor subunit mRNA (GluRs) expressed relative to the β-actin baseline were significantly greater than those in the respective aCSF control groups (aCSF-W2, aCSF-W4, aCSF-W8, aCSF-F2 and aCSF-F4) (Fig. 5a). Among the AMPA receptor subunits GluR1, GluR2, GluR3 and GluR4, only the quantity of GluR3 mRNA relative to β-actin was significantly increased in all of the KA-infused groups as compared with their respective aCSF control groups (KA-W vs. aCSF-W: 2 weeks, $39.6 \pm 3.8 \times 10^{-3}$ vs. $25.2 \pm 2.1 \times 10^{-3}$; 4 weeks, $41.8 \pm 4.6 \times 10^{-3}$ vs. $21.5 \pm 1.0 \times 10^{-3}$; 8 weeks, $44.8 \pm 3.8 \times 10^{-3}$ vs. $25.8 \pm 1.8 \times 10^{-3}$; KA-F vs. aCSF-F: 2 weeks, $33.8 \pm 1.2 \times 10^{-3}$ vs. $21.7 \pm 2.0 \times 10^{-3}$; 4 weeks, $46.3 \pm 2.2 \times 10^{-3}$ vs. $26.3 \pm 1.4 \times 10^{-3}$; mean ± SEM; $n = 5$ in each) ($p < 0.001$; Fig. 5a). Because the quantities of GluR1, GluR2 and GluR4 did not change in any of the KA-infused groups, the increase in total AMPA receptor subunit mRNA in these groups was caused by the selective increase in GluR3 mRNA. This molecular change resulted in a significant decrease in the proportion of GluR2

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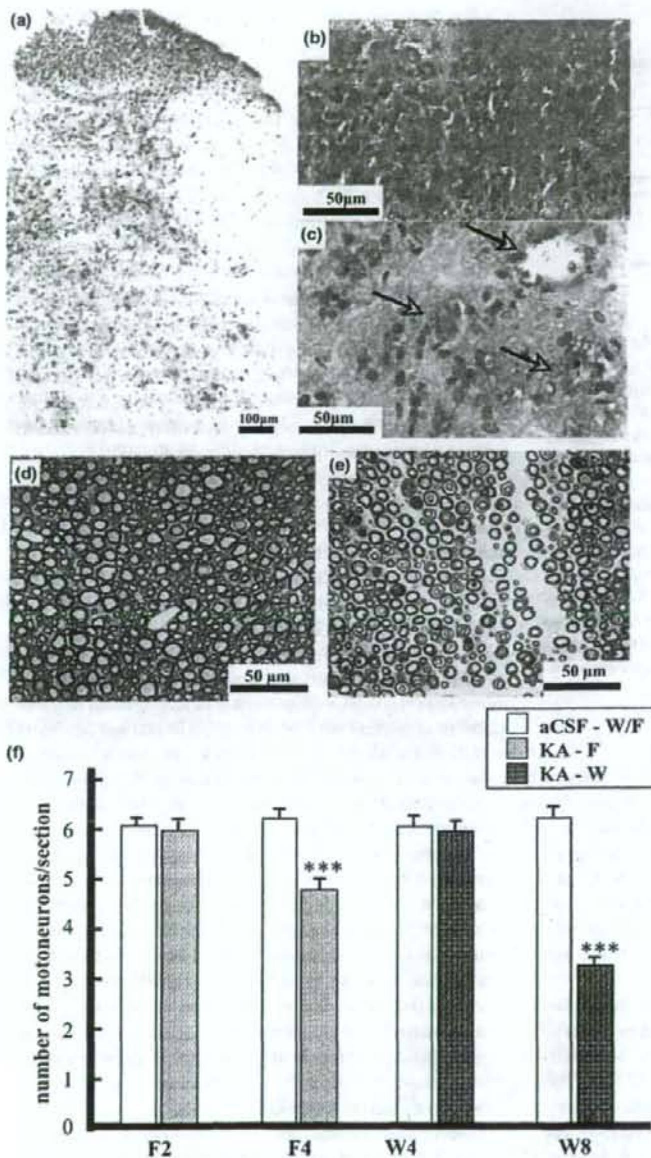

Fig. 2 Morphological changes in the kainic acid (KA)-infused rats. (a–c) Sections stained with hematoxylin and eosin demonstrate that motor neurons in the lumber spinal cords (L5 segment) of a KA-W8 rat exhibited severe morphological changes, including microvacuolation, loss of Nissl substance and glial margination, indicated by the arrows in panel (c), whereas neurons in the dorsal horn appeared intact (a and b). (d and e) Toluidine blue staining of an epon-embedded section of the L5 ventral root of an aCSF-W8 rat (d) and a KA-W8 rat (e) displaying axonal degeneration with a reduction in large myelinated fibers. (f) As compared with aCSF-infused controls, the number of motor neurons was not significantly different in KA-infused Fischer rats at 2 weeks (F2), but was significantly reduced at 4 weeks (F4) (***$p < 0.001$). Similarly, in the Wistar rats, the number of motor neurons was decreased between 4 weeks (W4) and 8 weeks (W8) in the KA-infused rats as compared with aCSF-infused control rats (***$p < 0.001$). The number of neurons with an identifiable nucleolus in a 20-μm-thick section of the L5 segment was expressed in each group. KA-W, KA-infused Wistar rats; KA-F, KA-infused Fischer rats; aCSF-W/F, aCSF-infused Wistar/Fischer rats.

mRNA relative to the total AMPA receptor subunit mRNA in all of the KA-infused groups (Fig. 5b). By contrast, there was no difference in the mRNA expression profile of AMPA receptor subunits including GluR3 mRNA between the KA-infused rats and their controls in either the substantia gelatinosa or the funiculus of the spinal cord (Figs 5c and d).

Discussion

The motor selective behavioral and neuropathological changes induced in this rat model are hallmarks of the clinicopathological changes seen in ALS. In addition, the delayed and progressive nature of these changes mimics ALS,

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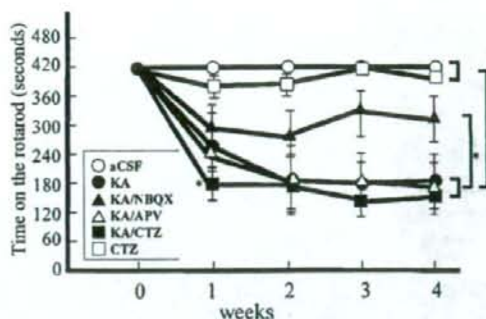

Fig. 3 Motor deficits in Fischer rats after co-infusion of kainic acid (KA) with various compounds. As compared with the KA-alone group, KA/6-nitro-7-sulfamobenso(f)quinoxaline-2,3-dione (NBQX)-infused rats exhibited significantly higher rotarod scores after 3 weeks of administration (* $p < 0.05$), whereas KA/APV-infused rats displayed the same level ($p > 0.1$). The rats co-infused with cyclothiazide (CTZ) and a lower concentration of KA (1.5 mm KA + 1.5 mm CTZ) displayed a more rapid decline in rotarod scores at week 1 as compared with those infused with a higher concentration of KA alone (3 mm KA) (* $p < 0.05$). In addition, infusion of CTZ alone (1.5 mm CTZ) slightly and only transiently decreased the rotarod score as compared with the aCSF-infused control. ●, KA-infused rats; ▲, KA/NBQX-infused rats; △, KA/APV-infused rats; ■, KA/CTZ-infused rats; □, CTZ-infused rats; ○, aCSF-infused rats. The rotarod speed was 16 rpm. Each value plotted indicates the mean rotarod score; error bars indicate the SD. *** $p < 0.001$ KA vs. aCSF and KA/CTZ vs. CTZ.

suggesting that the KA-infused rat is a clinicopathologically appropriate model for ALS. The fact that motor deficit preceded the neuropathological changes by between 2 and 4 weeks may indicate the dysfunction of motor neurons prior to death and the slow death-inducing process. In addition, the results with Wistar and Fischer rats indicate that it takes around 4 weeks to induce the death of motor neurons by this method, and the motor neuron vulnerability is slightly different among the rat strains.

Our co-infusion experiments strongly suggest that the neuronal degeneration observed was mediated by AMPA receptors, in agreement with the results of KA toxicity in cultured rat hippocampal neurons (Ohno *et al.* 1997). The observation that co-infusion of CTZ exacerbated KA toxicity lends further support to a mechanism of AMPA receptor-mediated neurotoxicity. CTZ is a desensitization blocker of AMPA receptors, particularly of the flip splice variants (Partin *et al.* 1994), and does not exhibit neurotoxicity by itself as is observed in cultured neurons (May and Robison 1993; Brorson *et al.* 1995) but enhances KA-induced neurotoxicity with a leftward shift of the KA dose–response curve in cultured neurons (Ohno *et al.* 1998). Because both the flip and flop variants of each AMPA receptor subunit are expressed in rat spinal motor neurons (Tölle *et al.* 1993), CTZ probably exacerbates the neurotoxicity of KA via its desensitization effects on AMPA receptors.

We and other researchers have investigated the neurotoxic effects of various glutamate receptor agonists on spinal neurons *in vivo* and found that glutamate receptor agonists induce the degeneration of different subsets of spinal neurons depending on the route and the duration of administration (Kwak *et al.* 1992; Nakamura *et al.* 1994; Kwak and Nakamura 1995a,b; Hirata *et al.* 1997; Corona and Tapia 2004). Although intrathecal infusion of KA for a brief period induces long-standing damage in spinal motor neurons, it also severely affects interneurons (Kwak and Nakamura 1995b). Because interneurons appeared to be intact after two months of intrathecal KA infusion in the present study, it is likely that in accordance with the results in the cultured neurons (Terro *et al.* 1998), the subtoxic dose of KA that we used here may have selectively activated motor neurons without non-specifically activating interneurons.

An increase in intracellular Ca^{2+} concentration owing to an influx through Ca^{2+}-permeable AMPA receptors has been demonstrated to play a pivotal role in AMPA receptor-mediated neuronal death in cultured motor neurons (Carriedo *et al.* 1996; Van Den Bosch *et al.* 2000). It is likely therefore that long-term administration of KA will induce an increase of Ca^{2+} influx through AMPA receptors, thereby causing the death of motor neurons. Here, we found that the expression of GluR3 mRNA expression was persistently increased in motor neurons, at least from week 2 of KA infusion when no motor neuron death was detected. Up-regulation of GluR3 mRNA was observed only in motor neurons and not in either dorsal horn neurons or in white matter, which morphologically remained intact throughout the experimental period, suggesting that this molecular change is probably caused by the long-term activation of motor neurons by KA. An increase in GluR3 mRNA has been reported in the motor neurons of mice transgenic for mutated human *SOD1* (SOD1G93 A mice) (Spalloni *et al.* 2004), and these motor neurons display an increased vulnerability to excitotoxicity (Spalloni *et al.* 2004). In addition, the survival of these mice can be prolonged by the administration of GluR3 antisense protein nucleic acid (Rembach *et al.* 2004). These findings suggest that an increase in GluR3 mRNA in motor neurons is tightly associated with the mechanism underlying the selective degeneration of motor neurons.

GluR2 plays a critical role both in controlling the assembly and trafficking of AMPA receptors in hippocampal neurons (Sans *et al.* 2003), and in regulating Ca^{2+} permeability (Hollmann *et al.* 1991; Verdoorn *et al.* 1991; Burnashev *et al.* 1992). Furthermore, cultured neurons derived from GluR2-deficient mice are more vulnerable to excitotoxicity than those derived from wild-type animals (Iihara *et al.* 2001; Van Damme *et al.* 2005), and mice deficient for GluR2 RNA editing at the Q/R site die young as a result of premature neuronal death (Brusa *et al.* 1995). Therefore, an

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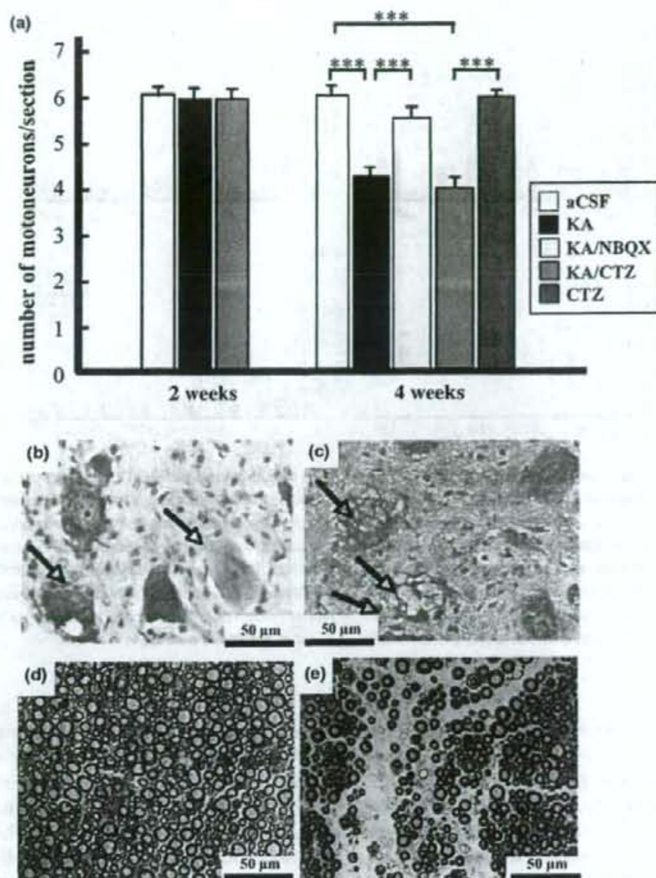

Fig. 4 Effects of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-acting compounds on kainic acid (KA)-induced neurotoxicity in Fischer rats. Co-infusion of 6-nitro-7-sulfamobenso(f)quinoxaline-2,3-dione (NBQX) rescued the neuropathological changes in motor neurons of KA-infused Fischer rats (KA-F), whereas co-infusion of cyclothiazide (CTZ) worsened the changes. (a) The number of motor neurons was not decreased by week 2 in all the groups, whereas it was significantly decreased by week 4 in KA- and KA/CTZ-infused groups. The reduction in the number of motor neurons in KA-F4 was significantly ameliorated by co-infusion of NBQX (***$p < 0.001$). The number of motor neurons in the CTZ alone group was the same as that in the artificial cerebrospinal fluid (aCSF) control, whereas that in the KA/CTZ-infused group was significantly less than in the aCSF control (***$p < 0.001$) but was the same as that in the KA-alone group ($p > 0.1$). KA, KA-infused rats; KA/NBQX, KA/NBQX-infused rats; KA/APV, KA/APV-infused rats; KA/CTZ, KA/CTZ-infused rats; CTZ, CTZ-infused rats; aCSF, aCSF-infused rats. (b and c) Motor neurons in a rat infused with KA (1.5 mM)/CTZ (1.5 mM) for 4 weeks (c) exhibited more severe morphological changes as compared with motor neurons in a rat infused with KA (3 mM) for the same period (b), including marked cytoplasmic vacuolation with condensation of Nissl substance (arrows). (d and e) A ventral root from a KA/CTZ-infused rat at week 4 (e) displayed marked axonal degeneration with loss of fibers as compared with a ventral root from an aCSF-infused control rat at week 4 (d).

increase in GluR3 mRNA may result in a reduction in the relative proportion of GluR2 among AMPA receptor subunits, thereby increasing the proportion of GluR2-lacking, Ca^{2+}-permeable AMPA receptors among the functional AMPA receptors expressed in the motor neurons of KA-infused rats (Fig. 5b). Indeed, the survival of human *SOD1*G93 *A* transgenic mice can be prolonged by the overexpression of GluR2 (Tateno *et al.* 2004), whereas mice that are additionally deficient for GluR2 show decreased survival as compared with those transgenic for human *SOD1*G93 *A* alone (Van Damme *et al.* 2005). Thus, it seems likely that long-term infusion of KA induces the degeneration of motor

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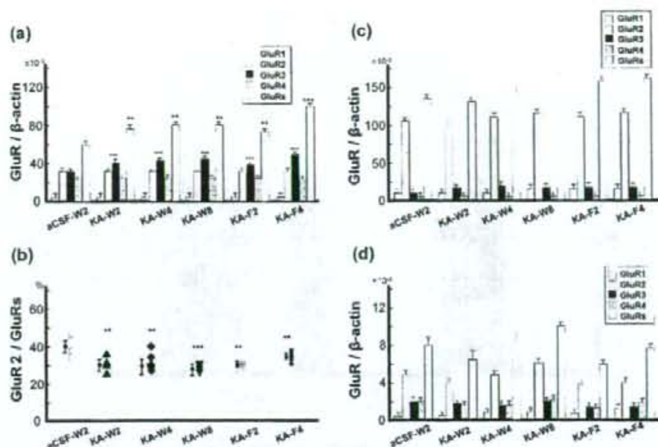

Fig. 5 Quantitative measurement of the expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit mRNA. (a) In motor neurons of each kainic acid (KA) group, the quantity of total AMPA receptor subunit mRNA and that of GluR3 mRNA relative to the β-actin baseline were significantly greater than those in their artificial cerebrospinal fluid (aCSF) controls (only aCSF-W2 is illustrated for simplicity) (**$p < 0.01$, ***$p < 0.001$). The expression of GluR1, GluR2 or GluR4 in each KA group did not differ from that in the respective aCSF control group. (b) The proportion of GluR2 mRNA relative to the total AMPA receptor subunit mRNA was significantly decreased in the motor neurons of each KA group as compared with their respective aCSF control group (**$p < 0.01$). (c and d) There was no difference in the mRNA expression profile of AMPA receptor subunits between the KA-infused rats and their respective controls in either the substantia gelatinosa (c) or the funiculus of the spinal cord (d).

neurons via an AMPA receptor-mediated mechanism by increasing the proportion of Ca^{2+}-permeable AMPA receptors in motor neurons; however, how KA increases GluR3 mRNA expression, or rather how KA selectively increases this expression in motor neurons while keeping that in dorsal neurons and white matter cells unchanged, remains to be elucidated. Because rat motor neurons express significantly lower quantities of GluR2 mRNA and higher quantities of GluR3 mRNA, as compared with other neuronal subsets (Sun *et al.* 2005), the up-regulation of GluR3 mRNA that presumably reflects an increased level of GluR3 protein may lead to a more marked reduction in the proportion of GluR2, and in turn to a higher proportion of GluR2-lacking, Ca^{2+}-permeable AMPA receptors in motor neurons after KA infusion. The characteristics of motor neurons with a low relative abundance of GluR2 may explain, in part, the selective vulnerability of these cells to AMPA receptor-mediated excitotoxicity.

Another factor influencing the Ca^{2+} permeability of AMPA receptors is the status of GluR2 mRNA editing at the Q/R site. Recently, we have demonstrated that a significant reduction in RNA editing of GluR2 at the Q/R site occurs, in a disease-specific and neuronal class-selective manner, in the motor neurons of patients with sporadic ALS (Kawahara *et al.* 2004, 2006). In addition, the number of neurons was significantly reduced in the ventral horn of the spinal cord of mice transgenic for artificial Ca^{2+}-permeable GluR-B(N) minigenes (Feldmeyer *et al.* 1999; Kuner *et al.* 2005). It is likely that this molecular change is closely relevant to the pathoetiology of ALS; however, we did not find any reduction in GluR2 editing efficiency in the motor neurons of KA-infused rats in this study (data not shown). Therefore, although long-term activation of AMPA receptors *per se* induces the death of motor neurons both *in vitro* and *in vivo*, the underlying mechanism is probably an alteration in the relative proportion of AMPA receptor subunits, and not a reduction in RNA editing. In addition to GluR3 up-regulation (Spalloni *et al.* 2004), a lack of reduction in GluR2 RNA editing (Kawahara *et al.* 2006) suggests the participation of an AMPA receptor-mediated neuronal death mechanism similar to that present in rats with mutated SOD1-associated familial ALS (ALS1). In this respect, the KA-infused rat is a model for ALS1 rather than sporadic ALS. There seems to be different Ca^{2+}-permeable AMPA receptors that mediate neuronal death, including those caused by underedited GluR2 and those caused by a lack of GluR2. It seems likely that the underediting of GluR2 mRNA specifically seen in sporadic ALS motor neurons (Kawahara *et al.* 2004) is not caused by long-term AMPA receptor activation but rather by a defect intrinsic to motor neurons in patients with sporadic ALS.

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Acknowledgements

We thank Dr Jun Shimizu for valuable discussion. This study was supported, in part, by a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14017020, 15016030 and 16015228 to SK).

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ORIGINAL CONTRIBUTION

A Positron Emission Tomography Study on the Role of Nigral Lesions in Parkinsonism in Patients With Amyotrophic Lateral Sclerosis

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Background: Patients with amyotrophic lateral sclerosis (ALS) sometimes exhibit parkinsonism, but the lesion responsible for parkinsonism has not been extensively studied.

Objective: To test whether nigrostriatal system dysfunction is responsible for parkinsonism in ALS.

Design: From the 182 ALS patients who were admitted to our neurology ward during the past 10 years, we extracted all the patients who satisfied the criteria of both parkinsonism and ALS.

Setting: The University of Tokyo Hospital.

Methods: We conducted $[^{18}F]$L-dopa and $[^{11}C]$N-methylspiperone positron emission tomography and technetium Tc 99m hexamethylpropyleneamine oxime single-photon emission computed tomography studies on 5 patients with ALS manifesting overt parkinsonism.

Results: Two male and 3 female patients (average age, 63.2±5.8 years) had ALS for an average of 28.6±21.5 months and had parkinsonism for an average of 15.2±11.4 months. Features of their parkinsonism were characterized by outstanding bradykinesia without resting tremor or dementia. The results of positron emission tomography studies indicated normal nigrostriatal function, but those of single-photon emission computed tomography demonstrated decreased blood flow in the frontotemporal cortices.

Conclusion: It is likely that parkinsonism in ALS is due to cortical lesions rather than nigrostriatal dysfunction and that both symptoms are the clinical manifestation of frontotemporal dementia with motor neuron diseases, including classic ALS.

Arch Neurol. 2006;63:1719-1722

PATIENTS WITH AMYOTROPHIC lateral sclerosis (ALS) or motor neuron disease (MND) rarely exhibit clinically overt parkinsonism.[1] Neuropathological studies of ALS report changes in the extramotor systems, including the substantia nigra, but also note rare findings of Lewy bodies in the affected substantia nigra.[2] Neuroimaging studies have demonstrated a subclinical reduction of striatonigral dopaminergic systems in ALS patients without clinically overt parkinsonism.[3] Therefore, it is possible that nigrostriatal system dysfunction occurs in ALS, but is not responsible for parkinsonism. We conducted $[^{18}F]$L-dopa and $[^{11}C]$N-methylspiperone positron emission tomography (PET) scans and technetium Tc 99m hexamethylpropyleneamine oxime single-photon emission computed tomography studies on 5 patients who exhibited both parkinsonism and ALS to further clarify the neurophysical effects of these combined conditions.

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METHODS

PATIENTS

Among the 2485 inpatients in the neurology ward of the University of Tokyo Hospital during the past 10 years (1995-2005), 182 patients were diagnosed with probable or definite ALS according to the revised El Escorial criteria.[4] Ten of these 182 patients exhibited clinically overt parkinsonism, and 5 of these patients were studied with PET.

POSITRON EMISSION TOMOGRAPHY

Studies using $[^{18}F]$L-dopa and $[^{11}C]$N-methylspiperone PET were performed with a HEADTOME IV (Shimadzu, Kyoto, Japan). Fourteen transaxial images were obtained, with a 6.5-mm interval parallel to the orbitomeatal line. Final resolution of the reconstructed image was 7.5 mm in the transaxial direction and 9.5 mm in the axial direction at full width half maximum. Transmission scans to correct photon attenuation were carried out at the begin-

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Table 1. Patient Characteristics

Patient/ Sex/Age, y	Course of ALS	Initial Symptom	Clinical Symptoms of Motor Neuron Disease			Dementia
			BP	UMN	LMN	
1/F/61	5 y	Upper limb weakness	Yes	Yes	Yes	No
2/F/73	7 mo	Dysarthria	Yes	Yes	Yes	No
3/F/59	2.5 y	Gait disturbance (hesitation)	No	Yes	Yes	No
4/M/59	10 mo	Dysphagia	Yes	Yes	Yes	No
5/M/64	3 y	Dysarthria	Yes	Yes	Yes	No

Abbreviations: ALS, amyotrophic lateral sclerosis; BP, bulbar palsy; LMN, lower motor neuron; UMN, upper motor neuron

Table 2. Parkinsonism Characteristics in Patients

Patient	Bradykinesia	Rigidity	Pulsion	Tremor	Course	Levodopa Dose and Effect of Treatment
1	Yes	Yes	Yes	No	2 y	200 mg/d over 6 mo; slightly effective
2	Yes (hesitation)	Yes	Yes	No	4 mo	400 mg/d over 2 mo; no change
3	Yes (hesitation)	Yes	Yes	No	2.5 y	NA
4	Yes	Yes	Yes	No	6 mo	NA
5	Yes	Yes	Yes	No	1 y	200 mg/d over 6 mo; slightly effective

Abbreviation: NA, not applicable.

ning of each study with germanium 68/gallium 68 external rotating sources. [^{11}C]N-methylspiperone (1110 MBq) was injected intravenously and a static image was obtained over a 10-minute period, starting at 85 minutes after the [^{11}C]N-methylspiperone injection. The [^{18}F]L-dopa study was performed on a different day. One hundred twenty minutes after an intravenous injection of 370 MBq of [^{18}F]L-dopa, a 12-minute static image was taken. As previously described,[5] the tissue radioactivity in each region of interest was corrected by subtracting the nonspecific retention in the cerebellar hemisphere; retention values of the caudate and putamen were expressed as the ratio of radioactivity in each region to that in the cerebellum.

RESULTS

Demographic data of the patients are presented in **Table 1**. The 2 male and 3 female patients were 63.2±5.8 years of age (mean ± SD; range, 59-73 years), with a mean ALS duration of 28.6±21.5 months (range, 7-60 months) and mean parkinsonism duration of 15.2±11.4 months (range, 4-30 months). Three patients initially displayed bulbar palsy, 1 had weakness in the lower extremities, and 1 had a gait disturbance (frozen gait type). Parkinsonism appeared after onset of ALS symptoms in 4 patients and at the same time as ALS symptoms in 1 patient. All patients exhibited predominant upper and lower motor neuron signs, and 4 patients had additional bulbar symptoms. Lower motor neuron involvement was demonstrated by needle electromyogram in all patients. Parkinsonism was characterized by severe bradykinesia and moderate muscle rigidity without resting tremor. Four patients had severe disturbance in postural reflex with marked pulsion, 2 patients had outstanding frozen gait, and all 5 patients had moderate muscle rigidity; however, none of the patients had resting tremors. Three patients were treated with levodopa for 2 to 6 months without any significant beneficial effect on their parkinsonism (**Table 2**). None of the patients exhibited overt dementia, and all of them scored within normal ranges in the Mini-Mental State Examination and the Wechsler Adult Intelligence Scales–Revised. All patients exhibited normal brain magnetic resonance images. Both [^{18}F]L-dopa uptake and [^{11}C]N-methylspiperone binding were normal in all areas, including the caudal putamen (**Table 3** and **Figure**). Single-photon emission computed tomography demonstrated a decrease in the cortical blood flow in 4 of 5 patients (Table 3).

COMMENT

Our PET studies indicate that both preganglionic and postganglionic striatonigral dopaminergic systems were preserved in the patients who exhibited overt parkinsonism, in marked contrast to previous studies reporting that [^{18}F]L-dopa uptake was reduced in ALS patients without overt parkinsonism.[1] Clinical and neuropathological studies have presented findings from patients with parkinsonism associated with ALS, including, other than classic type, ALS with dementia, multisystem atrophy, postencephalitic ALS, diffuse Lewy body disease, and familial ALS.[2] Our ALS patients' clinical parkinsonism features were similar to those in the published literature but were atypical when compared with the typical features of Parkinson disease; all of these patients exhibited predominant akinesia, but none of them had exhibited resting tremor or considerable improvement after levodopa treatment. The relatively late age at onset and lack of dementia or autonomic dysfunction were common clinical features. There was a mild to moderate reduction in

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