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The Role of RNA Editing of Kainate Receptors in Synaptic Plasticity and Seizures

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Summary

The ionotropic glutamate receptor subunit GluR6 undergoes developmentally and regionally regulated Q/R site RNA editing that reduces the calcium permeability of GluR6-containing kainate receptors. To investigate the functional significance of this editing in vivo, we engineered mice deficient in GluR6 Q/R site editing. In these mutant mice but not in wild types, NMDA receptor–independent long-term potentiation (LTP) could be induced at the medial perforant path–dentate gyrus synapse. This indicates that kainate receptors with unedited GluR6 subunits can mediate LTP. Behavioral analyses revealed no differences from wild types, but mutant mice were more vulnerable to kainate-induced seizures. Together, these results suggest that GluR6 Q/R site RNA editing may modulate synaptic plasticity and seizure vulnerability.

Introduction

L-glutamate acts on three subtypes of ionotropic glutamate receptors: AMPA, NMDA, and kainate (Dingledine et al., 1999). AMPA receptors, assembled from GluR1-4 subunits, mediate the majority of fast excitatory neurotransmission in the central nervous system, while NMDA receptors are known to modulate synaptic plasticity. Glutamate receptors are also known to play a role in excitotoxicity under many pathological circumstances. The role of kainate receptors, assembled from GluR5-7, KA1, and KA2 subunits, is not as well understood (Lerma et al., 1997; Chittajallu et al., 1999; Frerking and Nicoll, 2000). There is evidence to suggest that kainate receptors modulate synaptic transmission by both pre- and postsynaptic mechanisms (Lerma et al., 1997; Chittajallu et al., 1999; Frerking and Nicoll, 2000) and that they can mediate synaptic plasticity at the mossy fiber–CA3 synapse (Bortolotto et al., 1999). Furthermore, substantial evidence indicates that kainate receptors may contribute to temporal lobe seizures (Nadler, 1981; Ben-Ari, 1985; Mulle et al., 1998; Bernard et al., 1999).

The properties of some glutamate receptor subunits are altered by RNA editing (Seeburg et al., 1998). The AMPA receptor subunit GluR2 and the kainate receptor subunits GluR5 and GluR6 undergo Q/R site RNA editing (Seeburg et al., 1998). This editing requires an intronic editing complementary site (ECS) that directs an enzymatic deamination in the pre-mRNA prior to splicing (Seeburg et al., 1998). The deamination results in substitution of a codon for glutamine (CAG) with a codon for arginine (CIG) in membrane domain 2 (M2). The arginine confers reduced calcium permeability (Egebjerg and Heinemann, 1993), reduced ion conductance (Swanson et al., 1996), and altered current/voltage relations (Bowie and Mayer, 1995; Kamboj et al., 1995) in the case of AMPA receptors containing edited GluR2 or kainate receptors containing edited GluR5 and/or GluR6 subunits.

While the mechanism of editing has been described, the biological significance of editing is unclear. Genetic alteration of the codon for Q to R at the GluR5 Q/R site revealed no obvious effects on kainate-induced seizures or behavior in mice (Sailer et al., 1999). Deletion of the ECS from the AMPA receptor GluR2 gene (Brusa et al., 1995; Feldmeyer et al., 1999) or ablation of the editing enzyme ADAR2 (Higuchi et al., 2000) in mice led to reduced RNA editing at the GluR2 Q/R site, altered AMPA receptor calcium permeability, NMDA receptor–independent LTP (Feldmeyer et al., 1999), and spontaneous seizures that were lethal (Brusa et al., 1995; Higuchi et al., 2000). This demonstrates that GluR2 editing is critical for survival. However, since GluR2 appears to be fully edited throughout development in rodents, it is unclear as to why editing evolved instead of a codon for R at the GluR2 Q/R site. Indeed, eliminating the requirement for editing by engineering a constitutive R at the GluR2 gene Q/R site had no obvious effects in mice (Kask et al., 1998) and was solely able to rescue the lethal phenotype observed in ADAR2 knockout mice (Higuchi et al., 2000). Thus, while it has been clearly demonstrated that there is an essential requirement for an R at the Q/R site in GluR2, the role of editing remains uncertain.

Unlike the case for GluR2, kainate receptor subunit Q/R site editing is subject to developmental regulation (Bernard and Khrestchatisky, 1994). The GluR5 and GluR6 subunits are unedited in the rat embryo, but editing rises to as high as about 55% and 85%, respectively, by birth. There is also regional regulation of the extent of kainate receptor editing in the brain, and significant alterations of kainate receptor editing occur following seizures in the human and the rat (Grigorenko et al., 1998; Bernard et al., 1999). This has led to the suggestion that the unedited kainate receptor subunits may play a role at excitatory inputs in the embryo and that the extent of editing at synapses may modulate synaptic transmission and seizure vulnerability in the adult. In order to investigate the role of unedited GluR6 and the biological role of editing in vivo, we genetically engineered mice

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deficient in GluR6 Q/R site editing. We report that unedited GluR6 subunits can mediate synaptic plasticity and that GluR6 editing in wild-type mice inhibits this mechanism of plasticity and reduces seizure susceptibility. The data provide evidence for a critical biological role of kainate receptor editing.

Results

Generation of Mutant Mice

Our aim was to eliminate GluR6 Q/R site editing in mice in order to investigate the role of GluR6 editing in vivo. In previous in vitro studies, GluR6 Q/R site editing was shown to depend on an ECS located in the intron between the exon encoding M2 and the exon encoding M3 of the GluR6 gene (Herb et al., 1996). Therefore, we used gene-targeting strategies to delete the ECS from the GluR6 gene in order to reduce GluR6 Q/R site editing in mice (Figure 1). Homozygous mutant mice were viable, and mean weight at 21 days did not differ significantly from wild-type littermates (average weights of the offspring were 10.8 ± 2.4 g [ECSΔ mutant, n = 7], 11.2 ± 1.6 g [heterozygote, n = 8], and 11.9 ± 2 g [wild-type, n = 7]). The mutant mice also appeared normal in a behavioral test battery (Figure 2) that assessed acoustic startle, prepulse inhibition of acoustic startle, spontaneous motor activity (Marks et al., 1985; Logue et al., 1997), and anxiety, using the elevated plus maze and the black–white box. There was also no effect of genotype in the accelerating rotarod task or the fear-conditioning task (Figure 2).

Selective Alteration of GluR6 Q/R Site Editing

We used an editing assay (Schiffer and Heinemann, 1999) to determine if the extent of Q/R site editing of GluR6 mRNA transcripts was altered as expected in the mutant mice (Figure 3). As predicted from previous studies (Bernard and Khrestchatisky, 1994; Bernard et al., 1999), wild-type animals partially edit GluR6 RNA at the Q/R site (hippocampus, 76.5% ± 3.8%, n = 3; whole brain, 76.1% ± 0.8%, n = 3). In contrast, editing was essentially abolished in homozygous mutant mice (hippocampus, 4.4% ± 1.0%, n = 3; whole brain, 4.7% ± 3.2%, n = 3) and was significantly reduced in heterozygotes (hippocampus, 37.0% ± 0.6%, n = 3; whole brain, 34.8% ± 2.8%, n = 3). These results confirm that the ECS is necessary for normal GluR6 Q/R site editing and indicate that the mechanism of editing demonstrated in vitro (Herb et al., 1996) also applies in vivo.

Other sites in glutamate receptor subunit RNAs undergo editing (Seeburg et al., 1998; Higuchi et al., 2000). We investigated if deletion of the ECS from the GluR6 gene led to alterations of editing at other positions that affect glutamate receptor calcium permeability. As shown in Figure 3, we found no significant alterations in our assays (Schiffer and Heinemann, 1999) of GluR5 Q/R site editing, GluR6 tyrosine to cysteine (Y/C) editing at amino acid 571, and GluR6 isoleucine to valine (I/V) editing at amino acid 567. Furthermore, no unedited AMPA receptor GluR2 subunit mRNA was detected at the Q/R site in either mutants or wild types. The data provide evidence that editing at different sites in GluR5, GluR6, and GluR2 mRNA are regulated independently

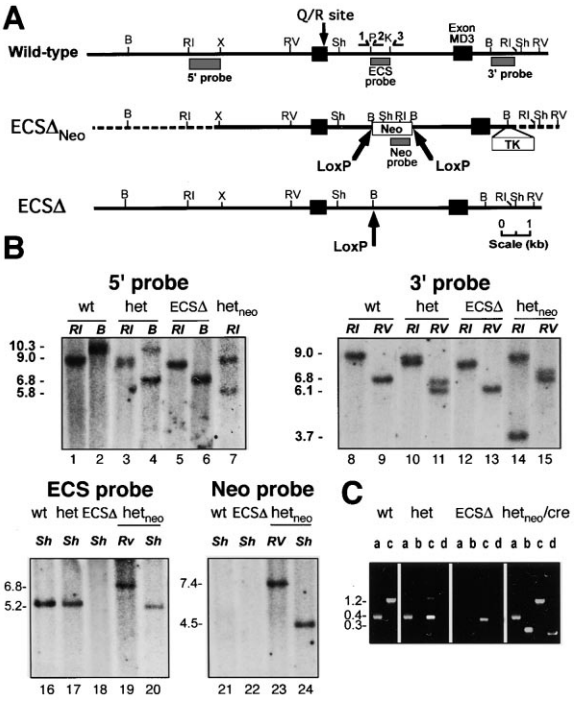

Figure 1. Generation of GluR6 Q/R Site Editing–Deficient Mice

(A) Restriction map of the GluR6 gene. (Wild-type allele) The exons encoding M2 and M3 of the GluR6 gene are shown as black boxes. The ECS probe is a PstI (P)/KpnI (K) fragment that contains the ECS (Herb et al., 1996). (ECSΔ_{Neo} allele) This allele was generated by homologous recombination in ES cells. The solid line indicates the gene-targeting construct. The NeoR gene replaced the PstI/KpnI fragment containing the ECS. The thymidine kinase (TK) gene (open box) was deleted during homologous recombination. (ECSΔ allele) In this allele, present in the mutant mice used in this study, a single loxP and BamH1 site remains (arrow) where the NeoR gene was excised by in vivo Cre recombinase-mediated recombination (see Experimental Procedures).

(B) Southern blot analysis. DNAs from wild-type (wild-type), ECSΔ heterozygote (heterozygous), and ECSΔ homozygote (ECSΔ) mice were analyzed as well as DNA from a mouse heterozygous for the ECSΔ_{Neo} allele and the Cre transgene (het$_{Neo}$/cre). In mice heterozygous for ECSΔ_{Neo}, the 5′ probe (Figure 1A) identified a 5.8 kb EcoRI (R1) fragment in addition to the wild-type 9 kb fragment ([B], lane 7). Likewise, the 3′ probe shown in (A) identified a 3.7 kb ECSΔ_{Neo} fragment ([B], lane 14). Following excision of NeoR, the EcoRI site is deleted and the wild-type pattern restored ([B], lanes 3, 5, 10, and 12). However, an internal BamHI (B) site introduced in the ECSΔ_{Neo} allele remained (A), generating a 6.8 kb band in ECSΔ- and ECSΔ_{Neo}-containing mice ([B], lanes 2, 4, and 6). EcoRV (RV) digestion revealed fragments of 6.8, 6.1, and 7.3 kb with the 3′ probe in mice, consistent with bands expected for the wild-type, ECSΔ, and ECSΔ_{Neo} alleles, respectively ([B], lanes 9, 13, and 15). As expected, the ECS probe did not hybridize in mice homozygous for the ECSΔ allele ([B], lane 18), while the NeoR probe only hybridized to DNA from mice with the ECSΔ_{Neo} allele ([B], lanes 23 and 24).

(C) PCR analysis. The primers used are shown in (A). PCR lanes are "a," primers 1 and 2 across the PstI site, showing loss of signal in mutants but not when the wild-type allele is present, "b," primers 1 and mcN994^{+} (specific for the Neo gene in Fig 1A) showing, as expected, a band only when the ECSΔ_{Neo} allele is present, "c," primers 1 and 3 across the PstI and KpnI sites, showing the expected sizes for the wild-type allele (1.2 kb) and ECSΔ allele (0.4 kb), and "d," PCR for the Cre recombinase transgene, showing a band only in hetNeo/cre mice. Sh, SphI; X, XbaI.

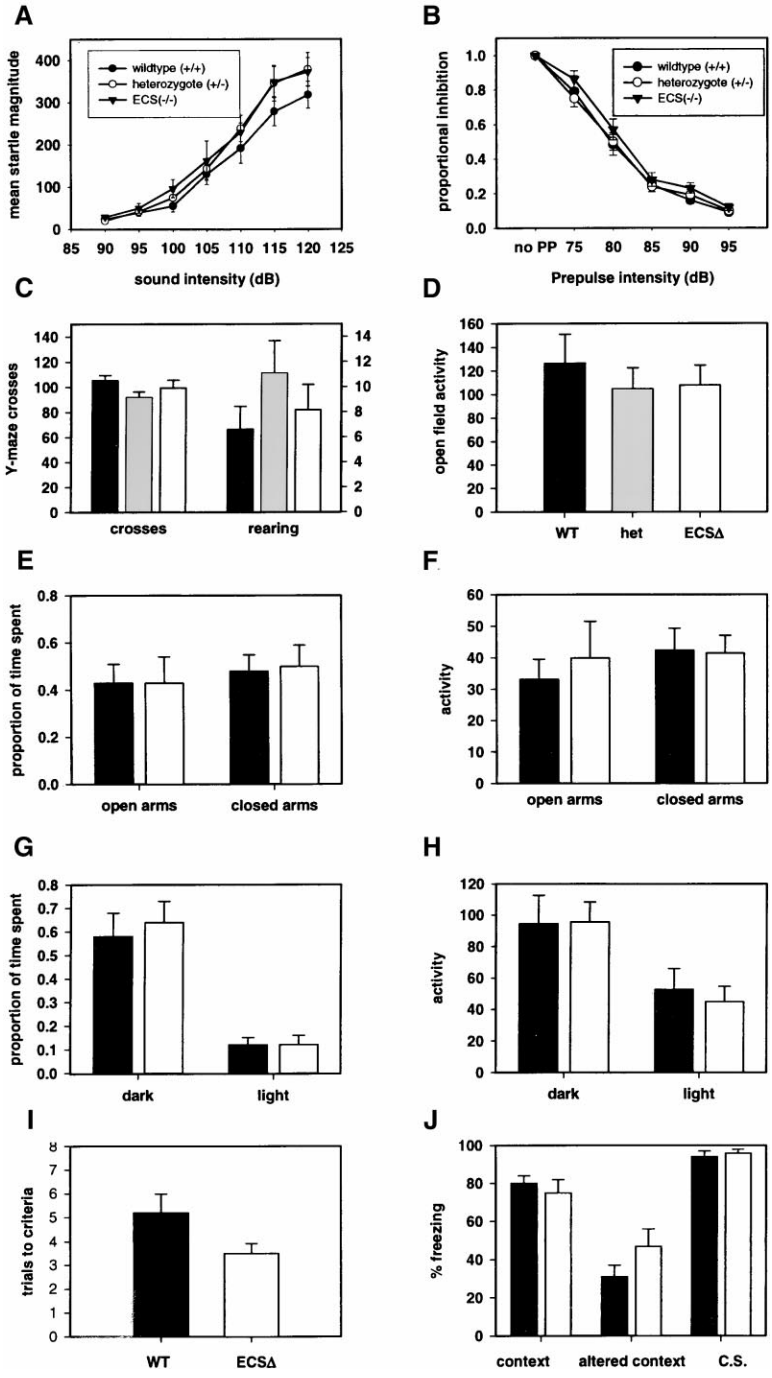

Figure 2. Behavioral Analyses

Black, wild types; gray, heterozygotes; and white, mutants. Data shown are averages ± SEM.

(A and B) Mean startle amplitude (A) and prepulse inhibition (B) for wild-type (closed circle), heterozygous (open circle), and ECSΔ mutant (closed triangle) mice (n = 16). Mice went through a 3 min acclimation period of 70 dB white noise followed by a sequence of pure-tone acoustic trials interspersed with PPI trials given in pseudorandom order. Each sequence consisted of one of each type of acoustic trial (90, 95, 100, 105, 110, 115, or 120 dB) and one of each type of PPI trial (acoustic prepulse of 75, 80, 85, 90, or 95 dB given 120 ms prior to a 120 dB acoustic stimulus). Each trial was separated by an intertrial interval of 10 or 15 s, and the entire sequence was repeated four times. Readings were collected beginning at stimulus onset (1 ms intervals), and the startle amplitude was defined as the average of 50 readings. The average of all four presentations of each trial was used, and the ratio of startle response with prepulse to response without prepulse was determined.

(C) Y maze activity. Animals were placed in a closed Y maze, and photobeam breaks, signifying Y maze crosses and rears, were recorded for 3 min. Wild-type, n = 16; heterozygous, n = 15; ECSΔ mutant, n = 16.

(D) Open-field activity. Animals were placed in a brightly lit circular open field, and photobeam breaks were recorded for 10 minutes. Wild-type, n = 15; heterozygous, n = 15; ECSΔ mutant, n = 16

(E) Elevated Plus Maze. Photobeam breaks were recorded in each compartment for 5 min (n = 8). The proportion of time spent in the open and closed arms was recorded

(F) Plus maze activity. Number of photobeam breaks in the open and closed arms was recorded (n = 8).

(G) Black–white box. Mice were started in the corner of the light compartment of the box, and photobeam breaks were recorded in each compartment for 5 min. Proportion of time in each compartment was determined. Wild-type, n = 11; ECSΔ mutant, n = 10.

(H) Black–white box activity. Number of photobeam breaks in each compartment was determined. Wild-type, n = 11; ECSΔ mutant, n = 10.

(I) Rotarod. The rotarod (Ugo Basile, Italy) was set to accelerate from 4 to 40 rpm over 5 min, and the time to fall off was recorded. The protocol was repeated approximately every 30 min for 10 trials, until the animals learned to stay on the rod for minimum of 4 min (n = 10).

(J) Fear conditioning. Eight male and eight female mice of each genotype were placed individually in a conditioning chamber for 2 min, and then they received two pairings of a clicker auditory cue with a 0.7 mAmp footshock. The next day, the percent time freezing was scored when animals were returned to (1) the conditioning chamber (context) for 5 min by scoring the absence or presence of freezing every 10 s. (2) Later (1 hr), each mouse was placed in a modified chamber in which the size and the tactile and olfactory cues were changed to provide the altered context and scored for freezing over a 3 min period. (3) The clicker auditory cue (conditioning stimulus [C.S.]) was then presented and mice scored for freezing for an additional 3 min. Scores did not differ significantly between (3) and (1).

of GluR6 Q/R site editing. This is consistent with the dependence of RNA editing on intron sequences.

Three assays were performed to confirm that the ECS deletion did not affect the levels or patterns of GluR6 expression. First, in an RNase protection assay, the GluR6 mRNA expression was not reduced in mutant mice (Figure 4A; ECSΔ mutant:wild-type = 0.9 ± 0.1, n = 2). Second, in situ hybridization analysis indicated that GluR6 RNA expression patterns were not altered in mutant mouse brains (Figure 4B). Third, immunoblot

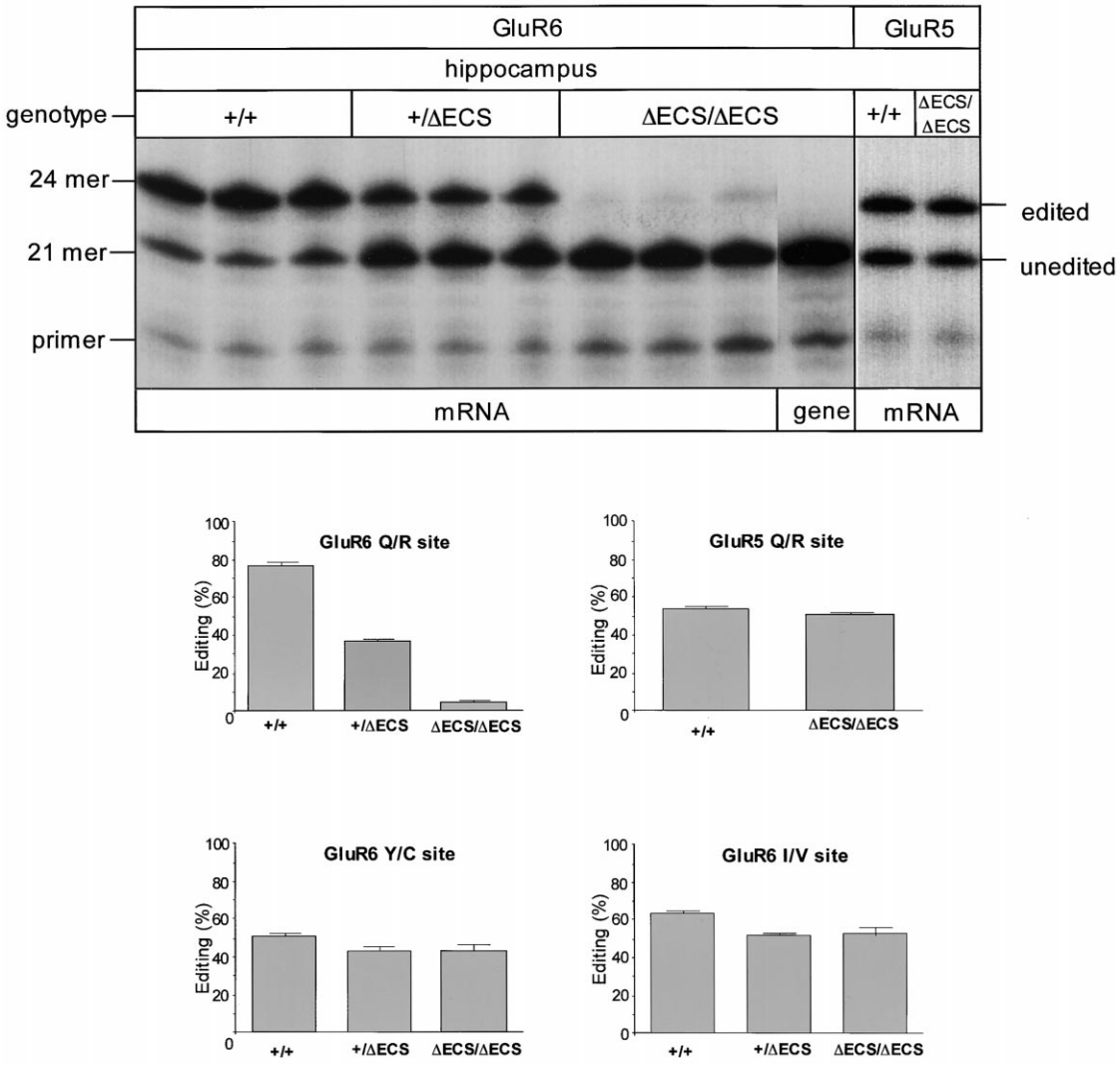

Figure 3. Selective Alteration of GluR6 Q/R Site Editing

Total mouse hippocampal RNA or genomic DNA was isolated from wild-type, heterozygote, and homozygote mutant mice. A short region around the editing site in the mRNA was amplified by RT–PCR. A primer extension was then performed on this template in the presence of ddATP. Incorporation of ddATP caused the extension to terminate at the Q/R site if the codon was unedited (CAG), yielding a 21-mer product. If the template was edited, (CIG) however, synthesis continued to the next A, resulting in a 24-mer product. The ratio between the intensity of the shorter 21-mer and the longer 24-mer represents the ratio of unedited to edited mRNA. Percent editing was determined for hippocampal mRNA at the GluR6 Q/R site, the GluR5 Q/R site (ECSΔ mutant, 54.3% ± 1.17%; wild-type, 50.4% ± 1.4%; n = 3), the GluR6 Y/C site (ECSΔ mutant, 43% ± 1.9%; heterozygous, 42.9% ± 3.1%; wild-type, 50.2% ± 1.97%; n = 3), and the GluR6 I/V site (ECSΔ mutant, 52.4% ± 4.23%; heterozygous, 51.9% ± 0.77%; wild-type, 63.4% ± 1.28; n = 3), or in whole-brain mRNA at the GluR5 Q/R site (ECSΔ mutant, 56.4% ± 4.0%; wild-type, 61.9% ± 0.5%; n = 3; data not shown). As expected, the Q/R site in the GluR6 genomic DNA was completely unedited (Figure 2A). This editing assay has been previously described (Schiffer and Heinemann, 1999).

analysis (Figure 4C) demonstrated that GluR6 protein expression is unchanged in the mutant mice. Additional RNase protection assays indicated that the expression levels of GluR5 (ECSΔ mutant:wild-type = 1.0 ± 0.2, n = 2) or GluR2 (ECSΔ mutant:wild-type = 0.9 ± 0.1, n = 2) that can affect calcium permeability of kainate or AMPA receptors, respectively, also remained unaltered (Figure 4A).

GluR6 Q/R Site Editing Determines Properties of Kainate Receptors

Editing at the GluR6 Q/R site determines the electrophysiological properties of kainate receptors in heterologous expression systems (Bowie and Mayer, 1995; Kamboj et al., 1995). We investigated whether current/voltage relationships were also changed in neurons cultured from the hippocampus of the mutant mice. All seven neurons analyzed from two GluR6 ECSΔ mutant mice showed rectifying kainate receptor responses, recorded in the presence of GYKI 53655 and AP-V, to block AMPA receptors and NMDA receptors, respectively (Figure 5A). This is consistent with responses expected from kainate receptors containing unedited subunits. By contrast, out of 12 neurons from two wild-type mice, 11 showed linear kainate receptor current/voltage characteristics (Figure 5C), and one showed a rectifying

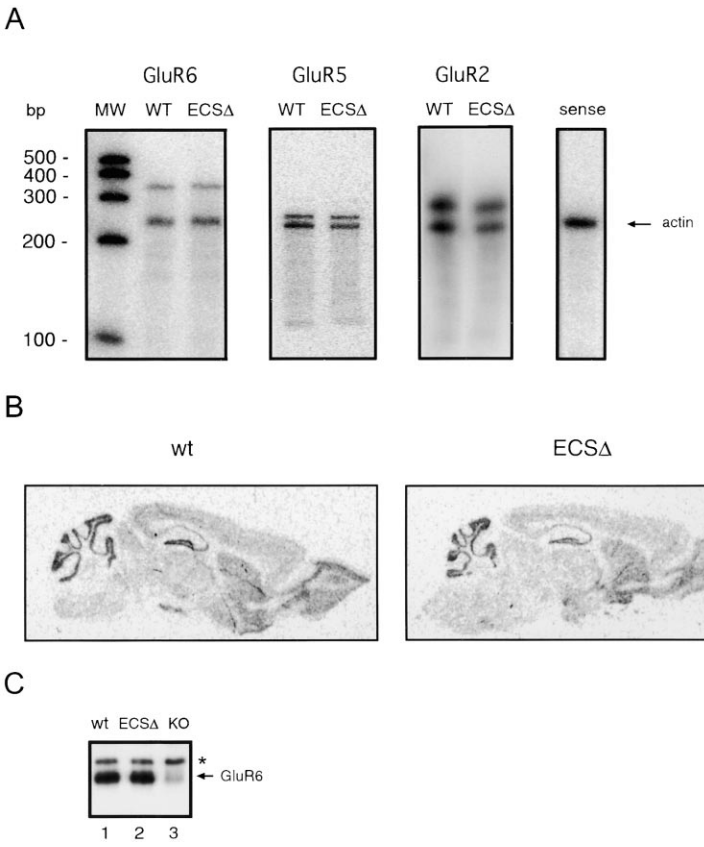

Figure 4. Normal Expression of GluR5, GluR6, and GluR2

(A) RNase protection analysis. Representative experiments are shown. In each lane, the upper band is the protected GluR6, GluR5, or GluR2 antisense ^{32}P-labeled RPA probe, and the lower band is a ^{32}P-labeled antisense actin probe, included as an internal standard (arrow). One representative control is shown, demonstrating, as expected, total degradation of a sense RPA probe, while actin is still protected. A non-RNase-treated sample was analyzed in parallel for each probe to confirm the expected size reduction of the protected species due to removal of the nonhomologous 5′ and 3′ overhangs of the RPA probe (data not shown).

(B) In situ hybridization. Parasagittal sections of brains from wild-type or ECSΔ mutant mice were hybridized with ^{33}P-labeled GluR6 5′ mRNA as previously described (Mulle et al., 1998).

(C) Immunoblot analysis. Hippocampal membrane protein (10 μg) extract from wild-type, ECSΔ mutant, and GluR6 null mutant mice (Mulle et al., 1998) was analyzed using an antibody directed at GluR6 and GluR7 (Raymond et al., 1993). The arrow indicates a band of relative molecular mass of ~115,000 Da, which is greatly reduced in intensity in the GluR6 null mutant. The residual band is attributable to GluR7 expression. The upper band (asterisk) represents nonspecific cross reactivity of the secondary antibody, as it is present in wild-type extract without primary antibody (data not shown) (Mulle et al., 1998).

response (data not shown), consistent with a low frequency of unedited GluR6 in neurons from wild-type mice (Ruano et al., 1995). Thus, kainate receptor responses are altered in hippocampus of mutant mice compared to responses in wild-type neurons. As expected, AMPA receptor responses determined in the absence of GYKI 53655 were linear in neurons from mutant and wild-type mice (Figures 5B and 5D) (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh and Jonas, 1995).

We also used calcium imaging to determine if kainate receptor currents were altered in the mutant mice. Following bath application of kainate in the presence of GYKI 53655 and AP-V, hippocampal neurons of the mutant mice showed increased calcium levels compared with responses observed in the neurons of wild-type mice (Figures 5E and 5F). This indicates that kainate receptors in the ECSΔ mutant mice have an increased calcium permeability compared to receptors in the wild-type mice.

GluR6 Q/R Site Editing Modulates Long-Term Potentiation

Since a high level of GluR6 subunit expression is seen in dentate gyrus granule cells (Egebjerg et al., 1991; Wisden and Seeburg, 1993), we decided to study kainate receptor function at this synapse. Long-term potentiation (LTP) at the medial perforant path synapse onto dentate gyrus granule cells is mediated postsynaptically by calcium influx through NMDA receptors (Christie and Abraham, 1992; Colino and Malenka, 1993). We hypothesized that calcium influx through kainate receptors containing unedited GluR6 at this synapse may be sufficient for induction of NMDA receptor–independent LTP (Bortolotto et al., 1999).

In order to test this idea, we studied LTP induction at this synapse in hippocampal slices either in the absence or in the presence of the NMDA receptor antagonist AP-V. As expected (Christie and Abraham, 1992; Colino and Malenka, 1993), we observed LTP induction in slices obtained from wild-type and mutant mice in the absence of AP-V (Figure 6A). Also as seen previously (Christie and Abraham, 1992; Colino and Malenka, 1993), there was no NMDA receptor–independent LTP in slices from wild-type animals determined in the presence of AP-V (50 μM) (Figure 6B). However, in slices from the GluR6 ECSΔ mutant mice, the EPSP slope following a high frequency stimulation was altered by 27% ± 5% ($t[13] = 2.7$, $p = 0.018$) in the presence of AP-V (50 μM). The result shows that NMDA receptor–independent LTP can be mediated by GluR6-containing kainate receptors at the medial perforant path synapse onto dentate gyrus granule cells in GluR6 Q/R site ECSΔ mutant mice. This suggests that RNA editing of kainate receptors can regulate the induction of LTP at this synapse.

Altered Seizure Susceptibility in Mutant Mice

Kainate-induced seizures in mice are a widely used model of temporal lobe seizures in humans (Nadler et al., 1978). Previous work from our group has shown that

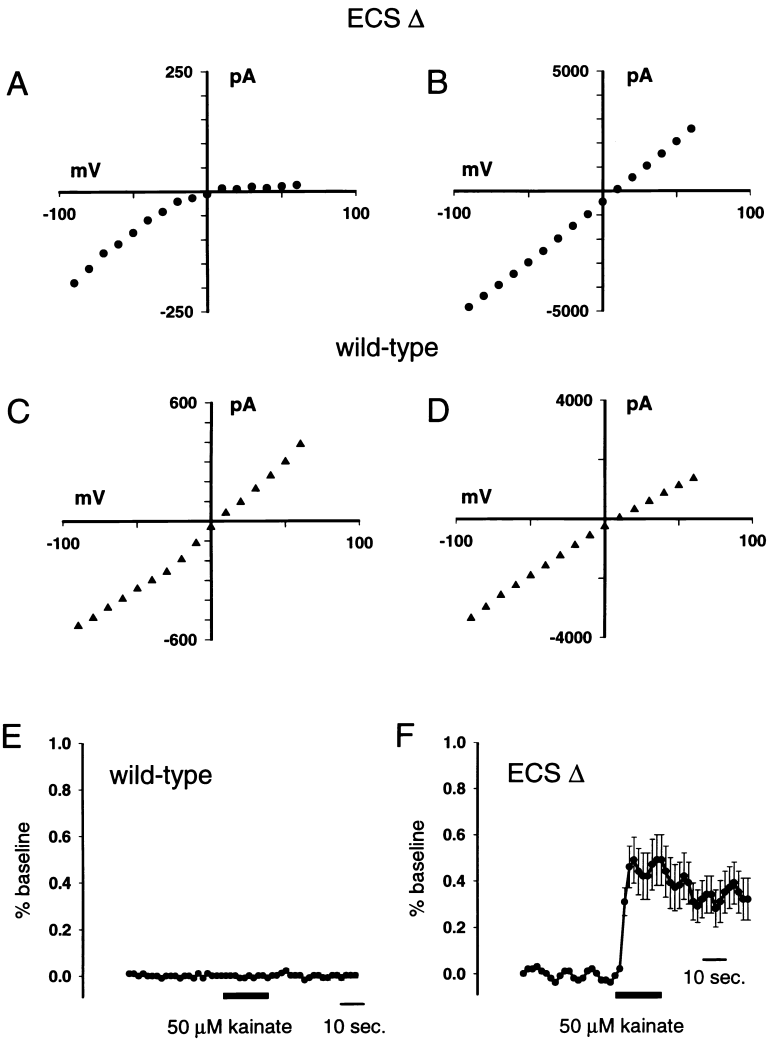

Figure 5. Kainate Receptor Responses Are Altered in Mutant Mouse Neurons

(A–D) Current/voltage (I/V) relationships of neuronal receptors. These were determined by electrophysiological patch-clamp recordings from cultured neurons 4 days after plating (Ghetti and Heinemann, 2000). Kainate was rapidly applied to the hippocampal neurons in vitro, and the peak current was recorded at holding potentials of −100mV to +70mV. The resulting I/V relations are shown for a representative experiment on hippocampal neurons from (A) mutant mice in the presence of the AMPA receptor antagonist GYKI 53655 (GYKI), (B) mutant mice in the absence of GYKI, (C) wild-type mice in the presence of GYKI, and (D) wild-type mice in the absence of GYKI.

(E–F) Calcium imaging. Kainate was bath applied to hippocampal neurons in the presence of GYKI (100 μM) from (E) wild-type mice (n = 30) or (F) ECSΔ mutant mice (n = 32), showing increased calcium influx through mutant kainate receptors. Neurons were incubated for 30 min at room temperature in the presence of 3 μM Fura2-AM and 0.001% Pluronic F-127 (Molecular Probes) and imaged as previously described (Ghetti and Heinemann, 2000) prior to and during application of kainate. Included in the bath solution was 300 μg/ml concanavalin A to prevent kainate receptor desensitization (Partin et al., 1993) as well as 1 μM TTX, 50 μM AP-V, 100 μM $CdCl_2$, and 100 μM $NiCl_2$ to block calcium channels. In control experiments, application of high-potassium solution in the absence of $CdCl_2$ and $NiCl_2$ resulted in calcium influx in neurons from both mutant and wild-type, due to calcium influx through voltage-activated calcium channels (data not shown).

the GluR6 subunit determines the vulnerability of mice to kainate-induced seizures (Mulle et al., 1998), and the GluR6 Q/R site has been hypothesized to affect seizure vulnerability (Grigorenko et al., 1998; Bernard et al., 1999). We tested this hypothesis in the GluR6 ECSΔ mutant mice and found that, indeed, GluR6 editing–deficient mice showed a greater susceptibility to kainate-induced seizures compared to their wild-type littermate controls. At a dose of 20 mg/kg injected kainate, all GluR6 editing–deficient mice had convulsions, compared with 83% of heterozygotes and 33% of wild types (n = 12 per genotype) (Figure 7). All mice that underwent convulsions showed at least one severe convulsion of at least 20 s duration, with uncontrolled paw movements, loss of balance, some frothing at the mouth, and severe clonic convulsions including uncontrolled fitting. The time to the first seizure showed no significant difference between groups. Since GluR6 Q/R site editing is ~76.5%, 37.0%, and 4.4% in wild-types, heterozygotes, and mutant mice, respectively (see Figure 2B), the vulnerability to kainate-induced seizures is inversely correlated with the extent of GluR6Q/R site editing in mice (33%, 83%, and 100%, respectively; Figure 7).

Discussion

We used a genetic approach to generate mice with unedited GluR6 subunits in order to investigate the role of GluR6 Q/R site editing in mice. Unlike GluR2 Q/R site RNA editing, RNA editing of the GluR6 gene transcript is developmentally regulated (Bernard and Khrestchatisky, 1994; Seeburg et al., 1998). Thus, study of GluR6 editing–deficient mutant mice allows investigation of the role that the completely unedited GluR6 transcripts may play in wild-type embryos and the possible function of the 15%–30% unedited GluR6 transcripts found in the adult brain (Bernard and Khrestchatisky, 1994; Schmitt et al., 1996; Bernard et al., 1999). Furthermore, comparisons of wild-type and mutant mice provide direct insight into the biological role of RNA editing in development and its effects on receptor properties, synaptic function, and seizure susceptibility.

In our experiments, deletion of the intronic ECS from the GluR6 gene resulted in a 95% reduction in GluR6 Q/R site editing. Apart from altered GluR6 editing, no other changes in gene expression or RNA editing were observed that might affect I/V relations, calcium perme-

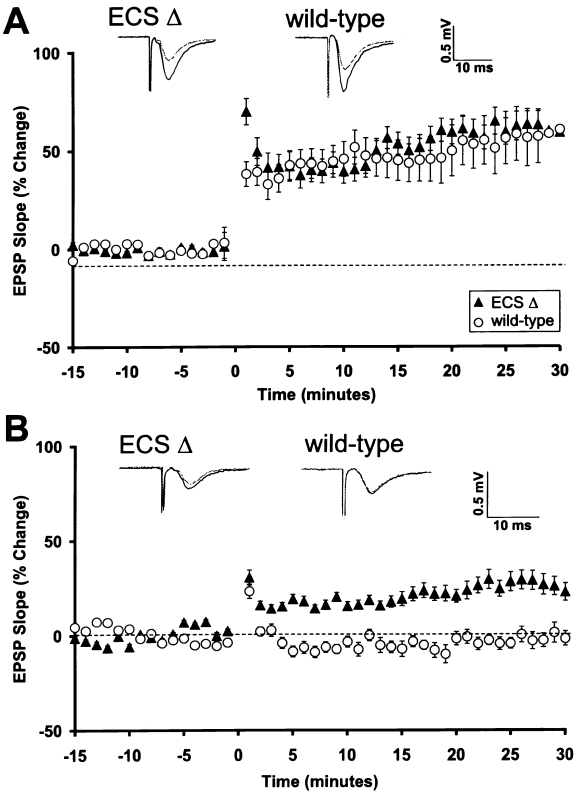

Figure 6. NMDA Receptor–Independent LTP in Mutant Mice

Field recordings of the medial perforant path–dentate granule cell synapse in hippocampal slices. LTP was induced by stimulation consisting of a burst of 50 pulses at 100 Hz, repeated four times at 30 s intervals.

(A) EPSP slope recorded without NMDA receptor antagonist. The result was averaged from 12 slices from 7 mutant and 10 slices from 6 wild-type mice. A significant degree of potentiation was observed in wild-type animals ($n = 10$; $67\% \pm 28\%$; $t(9) = 2.3$; $p < 0.05$) and in ECSΔ mutants ($n = 12$; $62\% \pm 13\%$; $t(11) = 4.9$; $p < 0.05$) following a tetanus.

(B) EPSP slope recorded with NMDA receptor antagonist. The result was averaged from 14 slices from 7 ECSΔ mutant and 11 slices from 6 wild-type mice. The slope of the evoked-field EPSPs in wild types did not show any significant change ($-1\% \pm 4\%$; $t(10) = 0.143$; $p = 0.889$). However, a significant degree of LTP was observed in the slices obtained from the mutant mice ($27\% \pm 5\%$; $t(13) = 2.7$; $p = 0.018$).

ability, or channel conductance of glutamate receptors. This demonstrates that, although editing of the I/V and Y/C sites in GluR6 and the Q/R sites in GluR5 and GluR2 requires ADAR2 (Higuchi et al., 2000), editing at these sites is regulated independently of the GluR6 Q/R site. We have therefore demonstrated the central and selective role of the GluR6 ECS for GluR6 Q/R site editing in vivo, confirming previous experiments that demonstrated its importance in vitro (Herb et al., 1996).

GluR6 Q/R site editing depends partially on the enzyme ADAR2. In an ADAR2 knockout mouse, a reduction of GluR6 Q/R site editing from 86% to 29% was observed, suggesting the other enzymes, for example, ADAR1, may be involved in GluR6 Q/R site editing (Higuchi et al., 2000). Since editing is reduced to ~5% in GluR6 ECSΔ mutant mice, these additional enzymes must also require the GluR6 ECS. The ~5% residual editing of GluR6 transcripts observed in the mutant mice suggests a minor mechanism of ECS-independent editing may also occur.

There was no observed change in GluR6 RNA or protein levels in GluR6 ECSΔ mutant mice. By contrast, GluR2 editing–deficient mice showed reduced GluR2 protein expression due to reduced splicing (Brusa et al., 1995). In those mice, the loxP sequence was inserted in close proximity to the edited exon, where the GluR2 ECS is located. It is not clear if editing and splicing mechanisms are independently affected by this loxP site or if the mechanisms are linked as has been suggested (Seeburg, 2000). However, the deleted intronic GluR6 ECS is distant from the edited exon, and the loxP site, which replaced it in GluR6 ECSΔ mutant mice, did not appear to affect GluR6 gene expression in our studies. This suggests that the GluR6 ECS is important for editing but not normal GluR6 gene expression.

Comparison of kainate receptor responses in hippocampal neurons from the GluR6 ECSΔ mutant and wild-type mice revealed that reduced GluR6 Q/R site editing results in increased calcium permeability and altered I/V relations. We found that expression and editing at other sites in GluR2, GluR5, or GluR6, which could affect the calcium permeability of glutamate receptors, were normal in the mutant mice. Our data therefore support the notion that regulation of editing at the GluR6 Q/R site alone can determine the calcium permeability and current/voltage characteristics of neuronal kainate receptors (Lerma et al., 1993; Ruano et al., 1995).

It has been demonstrated that both NMDA and AMPA receptors can mediate LTP via postsynaptic calcium influx through the receptor channel (Gu et al., 1996; Jia et al., 1996; Mahanty and Sah, 1998; Malenka and Nicoll, 1999). One report implicates kainate receptors in the induction of LTP at the mossy fiber–CA3 synapse by an unknown mechanism (Bortolotto et al., 1999). We studied LTP induction at the medial perforant path synapse onto dentate granule cells, where GluR6 is highly expressed (Egebjerg et al., 1991; Wisden and Seeburg, 1993). NMDA receptor–independent LTP was observed in the ECSΔ mutant mice but not in wild-type mice. This is evidence that GluR6 contributes to functional kainate receptors in the medial perforant path–dentate gyrus pathway. We also demonstrated that calcium-permeable kainate receptors can mediate LTP, although it is unclear from our data if it involves a pre- or postsynaptic mechanism.

A number of investigators have proposed that decreased GluR6 Q/R site editing may increase seizure susceptibility (Grigorenko et al., 1998; Bernard et al., 1999). We have demonstrated that vulnerability to kainate-induced seizures is inversely correlated with the extent of GluR6 Q/R site editing. Since kainate-induced seizures in mice are a model of temporal lobe seizures in humans (Nadler, 1981; Ben-Ari, 1985), our data support the notion, previously suggested, of a relationship between activation of kainate receptors, editing, neural plasticity, and epileptogenesis (Nadler, 1981; Ben-Ari, 1985; Mulle et al., 1998; Bernard et al., 1999; Sprengel et al., 1999). It is notable that GluR6 null mutant mice showed reduced susceptibility to kainate-induced seizures (Mulle et al., 1998). Since kainate selectively acti-

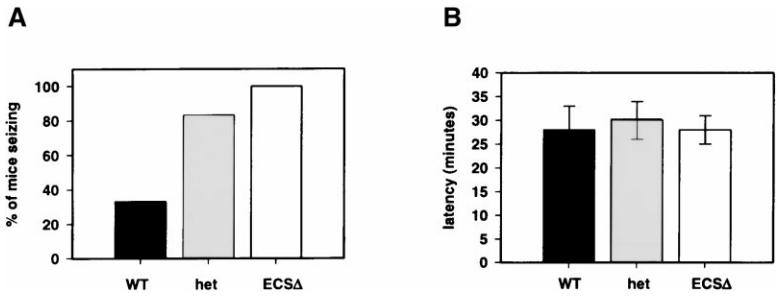

Figure 7. Increased Seizure Susceptibility in GluR6 ECSΔ Mutant Mice

Kainate-induced seizures. Kainate (20 mg/kg) was injected into 12 mice of each genotype.
(A) Percent mice showing a seizure following kainate injection.
(B) Latency to seizure following kainate injection (ECSΔ mutant, 28% ± 3%, n = 12; heterozygous, 30% ± 4%, n = 10; wild-type, 28% ± 5%, n = 4).

vates glutamate receptors, it was reasonable to conclude previously that GluR6 plays a critical and selective role in determining the animal's susceptibility to kainate-induced seizures. Taken together, our data suggest that editing regulates the degree to which GluR6 determines seizure vulnerability. This becomes relevant in the context of reports that show GluR6 editing is upregulated in human seizures, possibly as a mechanism to reduce seizure vulnerability (Grigorenko et al., 1998; Bernard et al., 1999).

Our results indicate that editing may play an important regulatory role in modulating kainate receptor properties, synaptic plasticity, and seizure vulnerability. The extent of editing rises from 0% embryonically to ~70%–85% in the adult (Bernard and Khrestchatisky, 1994; Schmitt et al., 1996; Bernard et al., 1999), varies between hippocampal neurons in cell culture (Ruano et al., 1995), and shows regional variation in the adult brain (Bernard and Khrestchatisky, 1994; Schmitt et al., 1996; Bernard et al., 1999). Recently, it was shown that AMPA receptors in single interneurons may have differing calcium permeability at different synapses (Toth and McBain, 1998). GluR6 Q/R site editing also provides the possibility for synaptic diversity. Since GluR6 is unedited embryonically, kainate receptors containing GluR6 may activate the same intracellular biochemical pathways in the embryo that mediate the NMDA receptor–independent LTP in the adult mutant deficient in RNA editing. This mechanism may be important to allow LTP induction in embryonic neurons or in adult neurons with unedited GluR6 when the NMDA receptor is absent or nonfunctional, as in nondepolarized neurons where the NMDA receptor is subject to magnesium block (McBain and Mayer, 1994). The ~15%–30% unedited GluR6 subunit RNA found in different brain regions (Bernard and Khrestchatisky, 1994; Schmitt et al., 1996; Bernard et al., 1999) could result in kainate receptors that enhance or directly mediate LTP at some synapses in wild-type adult mice. This might not be readily detected in field recordings in the dentate gyrus, since field recordings measure the summed responses of large populations of neurons, which would be dominated by the 85% edited GluR6 RNA in the adult dentate gyrus. However, in general, since ECSΔ mutant mice but not adult wild-type mice show kainate receptor–dependent plasticity at the synapse we studied, one effect of the developmental switch from unedited to mostly edited GluR6 could be to inhibit kainate receptor–mediated LTP at most synapses in adult wild-type mice.

Apart from the present study, only one other specific A to I editing site has been knocked out in an organism (Table 1). In studies from Seeburg's group (Brusa et al., 1995; Feldmeyer et al., 1999), the GluR2 Q/R site ECS was replaced with a Neo/TK selection cassette or a single loxP sequence. Using different combinations of these altered GluR2 alleles, mice were generated with different degrees of GluR2 gene expression and GluR2 Q/R site editing. This in turn affected the calcium permeability of AMPA receptors to varying degrees. In contrast to GluR6 ECSΔ mutant mice, varying degrees of altered GluR2 editing resulted in varying but severe phenotypes including hypomorphism, spontaneous seizures, and premature death. As with GluR6 ECSΔ mutant mice, GluR2 editing mutant mice showed no change in the extent of LTP induction but did demonstrate NMDA receptor–independent LTP. This finding contrasts with GluR2 knockout mice, which showed enhanced AMPA receptor calcium permeability that mediated both NMDA receptor–independent LTP and enhanced LTP but, surprisingly, no spontaneous seizures (Jia et al., 1996, B. V., unpublished data). This indicates that AMPA receptors with GluR2Q have unique effects compared to AMPA receptors that lack the GluR2 subunit, despite the fact that both flux calcium (discussed in Seeburg et al., 1998; Feldmeyer et al., 1999). Spontaneous seizures and death were also observed in ADAR2 knockout mice, which showed reduced GluR2, GluR5, and GluR6 Q/R site editing. However, reduced GluR2 editing but not reduced GluR5 or GluR6 editing accounted for this phenotype, since engineering an R in GluR2 alone rescued the ADAR2 knockout mouse from seizures and death. Our results are consistent with this finding, since GluR6 ECSΔ mutant mice showed no obvious cell loss in the hippocampus, behavioral phenotype, spontaneous seizures, or death. GluR6 ECSΔ mutant mice also showed no change in the time to seizure onset compared to wild types following kainate injections. However, they did show increased susceptibility to seizures induction, indicating that GluR6 editing modifies seizure vulnerability but not onset.

We have compared wild-type mice, which undergo extensive GluR6 Q/R site editing in the adult, with GluR6 Q/R site editing–deficient mice. We conclude that the switch from unedited to edited GluR6 in wild-type mice results in altered kainate receptor characteristics and inhibition of kainate receptor–mediated plasticity. This suggests a unique biological role for editing in modulating kainate receptor properties and synaptic plasticity during development and in the adult central nervous system. GluR6-editing mutant mice have increased vulnerability to kainate-induced seizures, suggesting that the developmental onset of GluR6 Q/R site editing may

Table 1. Mice with Alterations in Edited Glutamate Receptor Subunits

	AMPA Receptor	Kainate Receptor	
	GluR2	GluR5	GluR6
Knockout	• ↑ AMPA-R-mediated Ca^{2+} influx • enhanced LTP and NMDA-R-independent LTP at synapses onto CA1 pyramidal neurons[a] • no seizures described • reduced survival and altered behavior • Ref (Jia et al., 1996; Gerlai et al., 1998)	• abolishes kainate-R response in dorsal root ganglion cells • loss of kainate-R-enhanced neurotransmission at perforant path synapses onto hippocampal CA3 pyramidal cells[b] • no LTP effect found at mossy fiber–CA3 synapse • behavioral phenotype not yet reported • Ref (Contractor et al., 2000, 2001 [this issue of *Neuron*]; Mulle et al., 2000)	• ↓ postsynaptic kainate-R responses in CA3 pyramidal neurons • altered kainate-R responses in cerebellar golgi cells, striatal GABA-ergic projection neurons, and CA1 pyramidal cells[b] • ↓ LTP at mossy fiber–CA3 synapse • ↓ seizure vulnerablitly • no obvious behavioral phenotype • Ref (Mulle et al.,1998, 2000; Bureau et al., 1999, 2000; Chergui et al., 2000; Contractor et al., 2000, 2001)
Reduced Q/R site editing	• ↑ AMPA-R-mediated Ca^{2+} influx • NMDA-R-independent LTP at synapses onto CA1 pyramidal neurons[a] • spontaneous seizures • fatality depends on degree of editing • Ref (Brusa et al., 1995; Feldmeyer et al., 1999)	• not yet reported	• ↑ kainate-R-medicated Ca^{2+} influx • NMDA-R-independent LTP at synapses onto dentate granule cells[a] • ↑ seizure vulnerability • no apparent behavioral differences from wild-type • Ref (present study)
Constitutive R at Q/R site	• no apparent differences from wild-types • Ref (Kask et al., 1998)	• ↓ kainate-R response in dorsal root ganglion cells • no change in seizure vulnerability • no obvious behavioral phenotype • Ref (Sailer et al., 1999)	• not yet reported

[a] LTP at these synapses is NMDA receptor–dependent in wild-type mice.
[b] In addition, in mice lacking both GluR5 and GluR6, there is loss of kainate-R responses in CA1 stratum radiatum interneurons but not when either subunit is individually ablated. However, ablation of GluR6 alone results in loss of presynaptic KaR function between inhibitory interneurons (Mulle et al., 2000).

serve the critical function of reducing seizure vulnerability in adults. The increased seizure vulnerability in the GluR6 ECSΔ mutant mice is consistent with the idea that disregulation of editing in hippocampal neurons may be one mechanism underlying seizure generation (Grigorenko et al., 1998; Bernard et al., 1999; Sprengel et al., 1999) and suggests unedited GluR6 may contribute to the propensity for seizures in the developing brain (Johnston, 1996). Since the majority of childhood seizure syndromes resolve spontaneously (Sillanpaa et al., 1998), upregulation of kainate receptor editing during development may underlie their remission. Indeed, the role of GluR6 in seizure susceptibility and the absence of detectable behavioral changes in tests of both the GluR6 ECSΔ mutant and the GluR6 knockout mouse (Mulle et al., 1998) suggests GluR6 is a potential target for antiepileptic drugs.

Experimental Procedures

Generation of GluR6 Mutant Mice

The targeting construct (Figure 1) was electroporated into CCE embryonic stem (ES) cells (Robertson et al., 1986). One chimeric founder mouse was bred, generating isogenic 129/SvEv mice (Simpson et al., 1997) heterozygous for the $ECS\Delta_{Neo}$ allele (Figure 1). We removed NeoR by breeding with 129/SvJae PrmCre transgenic mice (O'Gorman et al., 1997). Offspring heterozygous for the ECSΔ allele (Figure 1) and lacking the PrmCre transgene (Figure 1C, lane D) were then interbred. The genetic background is a mixture of 129Sv/Ev and 129Sv/Jae (Simpson et al., 1997). Primers for PCR analysis in Figure 1: R6KO-1, CAAAGCTTAGTTAACTGATATACAG; R6KO-2, GCAAAGAGTGGGACATGGTGC. Primer 1, GAGTTCTCTCAGGTC TGAAGGGATACAC; primer 2, TACTCAAGGCAACGTCATGTCCAG CACT; primer 3, TGGCCCATCTTACACTTCAGTTCATCT; primer mcN, AATGGGCTGACCGCTTCCTCGTGCTT; and Cre primers, CTATCCAGCAACATTTGGGCCAGCTA and AACATTCTCCCACCG TCAGTACGTGA.

Editing Assay

A cycled primer extension editing assay was used (Schiffer and Heinemann, 1999). Total RNA was isolated from hippocampi or whole brains from 6- to 12-week-old mice (TRIZOL; Life Technologies, Rockville, MD). cDNA was synthesized (Thermoscript; Life Technologies) with gene specific primers complementary to the glutamate receptor 3′ untranslated regions. DNA was then amplified by PCR across the editing site, prior to the cycled primer extension assay.

RNAse Protection Assay

Templates for synthesizing probe were provided for actin (RPA-III kit; Ambion) or generated by PCR from cloned mouse cDNAs for GluR5, GluR6 (gifts from P. Gregor), and GluR2 (gift from M. Mishina) (GenBank accession numbers X66117, X66118, and X57498, respectively). The RPA probes we used were GluR6 5′ (353 bp), spanning the 5′ UTR and 5′ coding region; GluR5 5′ (270 bp), spanning the 5′ UTR and the 5′ coding region; and GluR2 exon 11 (297 bp), directed at the M2 region. The primers used to generate the RPA templates were GluR6, ACGACTCAGAATTCGGACTCCTTCCTCTT TCTGTGACCAT and GGTGACACTCTAGAAAGTCCTGTTCCTGTTG ATTGTATTC; GluR5, GGACGCCCGGAATTCGCTTGTTCCCTCCAC CTCGCTCTCCTG and GATCCTGAGCTCTAGAGGGGAGGTCTGA GGAAGGATGTAGCA; and GluR2, GCTGATATGAATTCTGCTCCAT TAACTATCACTCTCG and AAACCATCTAGACTTAAAAATCCCAAAT TCATTAGTT.

PCR products were cloned in pBluescript. RPA probes were tran-

scribed in vitro from the templates with α-32P-UTP using Ambion Maxiscript kit. Approximately 5 μg of hippocampal total RNA was used in the protection assays (RPA-III kit; Ambion). Protected products were quantified on a phosphoimager (Imagequant NT; Molecular Dynamics, Sunnyvale, CA). All results were normalized to actin controls.

Electrophysiological Analysis of Cultured Neurons

Rapid agonist application was performed using a three-barreled pipette (Ghetti and Heinemann, 2000). The central barrel was used to superfuse the cell continuously with the control solution containing 100 μM 1-(4-aminophenyl)-3-methylcarbamyl4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine; GYKI 53655; 1 μM TTX; 50 μM picrotoxin; and 0.01% fast green (Sigma). For activation of the kainate receptors, one of the lateral barrels was used to apply external solution containing 100 μM GYKI 53655, 300 μM kainate, 1 μM TTX, and 50 μM picrotoxin. Rectification index was determined as current at +50mV/current at −50mV.

Hippocampal Slice Electrophysiology

Brain slices (400 μm) were placed in artificial cerebral spinal fluid (ACSF; [pH 7.4], 32°C) containing bicuculline methiodide (1 mM) (Christie et al., 1996). A sharpened tungsten electrode was visually positioned in the middle third of the molecular layer to activate medial perforant path fibers. A 1 MΩ recording electrode filled with 1 mM NaCl was similarly positioned. Responses were evoked with single biphasic current pulses (10–400 mA) adjusted to yield responses ~30% of maximum. Evoked responses were initially tested using paired-pulse stimuli (at 50–500 ms). Only responses that did not show paired-pulse facilitation were used (McNaughton and Barnes, 1977). Maximal initial EPSP slope was measured using custom software (Lee Campbell; Getting Instruments). Each experiment was normalized to the values taken for the 10 min period prior to LTP induction.

Kainate Administration

Mice were injected intraperitoneally with 20 mg/kg kainic acid (Sigma) in saline and monitored for 2 hr for time of onset, occurrence, and severity of seizures.

Behavioral Tests

Mice were 5–6 months of age and were gender matched. Protocols and equipment were described previously (Marks et al., 1985; Logue et al., 1997; Wehner et al., 1997). The elevated plus maze is clear plexiglass (arms, 5 cm × 35 cm; the center, 5 cm^2). Two arms enclosed with 20.5 cm high walls a dim light (40 lux) overhead. The black–white box (26.7 cm high) has a red plastic lid. The black (15.2 × 26.6 cm) and white (31.5 × 26.6 cm) compartments are separated by a wall with a 7.6 cm square opening, and it has a light (20 lux) above the opening on the white side. Photoemitter-detector cells (Columbus Instruments; Columbus, OH) detected movement in the mazes. Analyses utilized the SPSS statistical package. Acoustic startle and PPI were analyzed by repeated measures multiple analysis of variance (MANOVA) and ANOVA. Y maze crosses and rears, open-field activity, body temperature, plus maze measures, and black–white box measures were assessed using one-way ANOVAs. Analyses were collapsed on sex because there was no interaction of sex with genotype for any variable examined. Individual mice were sometimes removed from the analyses due to equipment problems, where indicated. Alpha level was set at 0.05.

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