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Novel Toxicity of the Unedited GluR2 AMPA Receptor Subunit Dependent on Surface Trafficking and Increased Ca^{2+} -Permeability

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Abstract

RNA editing modifies the GluR2 AMPA receptor subunit pore loop at the Q/R site and limits receptor Ca^{2+} permeability. Editing failure is implicated in neurodegenerative diseases, including amyotrophic lateral sclerosis. We show that channels with unedited GluR2 are highly toxic in cultured hippocampal neurons. Toxicity exceeds that of other Ca^{2+} -permeable AMPA receptor types and is influenced by agonist binding site mutations, ability to desensitize, and extracellular Ca^{2+} levels. Significantly, toxicity also depends on GluR2's constitutive surface trafficking, a function dependent on GluR2 C-terminal domain interaction with NSF, a specialized chaperone. We have exploited the interaction between unedited GluR2 and NSF to reduce GluR2 surface levels. We show that a peptide that blocks the GluR2-NSF interaction reduces unedited GluR2 toxicity by reducing receptor surface expression. Peptide block of trafficking provides a model for design of drugs to reduce unedited GluR2 excitotoxicity in neurodegenerative diseases that result from editing failure.

Introduction

At CNS excitatory synapses, the activation of glutamate receptors induces depolarizing Na^+ and Ca^{2+} currents. Of the several glutamate receptor types, it is mainly NMDA receptors (NMDAR) that conduct Ca^{2+} in response to glutamate. However this induction of NMDAR Ca^{2+} currents is strictly controlled (Coan and Collingridge, 1985; Mayer et al., 1984). Under resting conditions, physiological levels of Mg^{2+} cause a block of NMDA receptors that is relieved when the postsynaptic neuron reaches depolarized potentials. The dual requirements for glutamate release and depolarization restrict NMDAR channel function to conditions of coincident pre and post-synaptic activity (Dingledine et al., 1999). Regulated influx of Ca^{2+} through the NMDAR can induce synaptic plasticity, including long-term-potential (LTP) and long-term depression (LTD), depending on the amplitude of Ca^{2+} signal (Malenka and Nicoll, 1993). Excessive activation of NMDARs can, however, trigger neuron death. Under anoxic conditions such as during stroke, neurons depolarize pathologically, promoting excessive release of glutamate and promiscuous NMDA receptor activation (Arundine and Tymianski, 2003; Choi, 1988). The resulting sustained high Ca^{2+} levels activate excitotoxic pathways that kill neurons.

The AMPA receptor (AMPA) subtype of glutamate receptors, unlike the NMDAR, does not require postsynaptic depolarization for its activation, and thus is activated under more

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permissive conditions (Herron et al., 1986). Nonetheless, AMPAR are, most commonly, not associated with excitotoxicity, largely because the prevalent AMPAR forms contain GluR2, which dominantly blocks Ca^{2+} permeability (Geiger et al., 1995; Spruston et al., 1995). AMPARs are composed of four subunits, GluR1, -2, -3 and -4, which are assembled into specific hetero or homotetramers (Hollmann and Heinemann, 1994) most of which, in hippocampus, contain GluR2 (Wenthold et al., 1996). The inclusion of one or more GluR2 subunits in the channel results in Ca^{2+} -impermeability (Seeburg et al., 1998). This ability of GluR2 to limit AMPAR Ca^{2+} conductance resides in the GluR2 pore forming loop when the pore loop structure has been modified by RNA editing (Seeburg et al., 1998). Editing of the structure of GluR2 mRNA by the RNA editing enzyme, ADAR2, an adenosine deaminase, changes the amino acid encoded at the apex of the loop from glutamine to arginine (Seeburg et al., 1998). Editing is normally highly efficient and the edited GluR2 dominantly restricts AMPAR Ca^{2+} conductance. GluR2-lacking AMPA receptors and those with unedited GluR2, however, are Ca^{2+} permeable.

Activation of Ca^{2+} permeable AMPAR can lead to abnormal synapse plasticity and to excitotoxicity. In GluR2 knockout mice, all AMPA channels display Ca^{2+} -permeability and these mice show unsaturable LTP (Jia et al., 1996). Notably, mice expressing unedited GluR2 (GluR2Q) or GluR2 with asparagine at the pore apex (GluR2N), both of which subunits fail to block Ca^{2+} conductance, show additional effects including epileptic seizures and early death (Feldmeyer et al., 1999; Kuner et al., 2005a; Kuner et al., 2005b). This suggests the GluR2 Ca^{2+} permeability associated with lack of RNA editing can have pathological consequences.

The trafficking of AMPA receptors to and from synapses regulates synapse strength and underlies LTP and LTD (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002). During the early phase of LTP, GluR1/2 heteromers or GluR1 homomers are incorporated into synapses by activity-dependent pathways. Later during the LTP mechanism, these GluR1 containing channels may be replaced by Ca^{2+} impermeable, GluR2/3-containing channels by an activity independent mechanism (Passafaro et al., 2001; Plant et al., 2006; Shi et al., 2001). AMPA receptors are assembled in the ER and traffic to and from synapses by subunit specific mechanisms, reliant on interactions of trafficking proteins with the subunit C terminal domain (CTD) (Barry and Ziff, 2002). The GluR1 CTD binds SAP97, a PDZ containing protein, while GluR1 is still in the ER and SAP97 is released upon receptor arrival at the synapse (Sans and Wenthold, 2001). GluR2 and GluR3 CTDs are significantly homologous and both bind the multi-PDZ proteins, GRIP (Glutamate Receptor Interacting Protein) (Dong et al., 1997) and ABP (AMPA Binding Protein) (Srivastava et al., 1998); (DeSouza et al., 2002). ABP/GRIP exist as palmitoylated and non-palmitoylated isoforms and may function as membrane anchors for AMPAR (Dong et al., 1997; Osten et al., 2000; Srivastava et al., 1998). The GluR2/3 CTDs also bind PICK1 (Protein Interacting with C Kinase1), a PDZ containing protein that contributes to AMPAR trafficking (Liu and Cull-Candy, 2005; Xia, 1999); (Barry and Ziff, 2002). GluR2 alone binds N-ethylmaleimide Sensitive Factor (NSF) (Nishimune et al., 1998; Osten and Ziff, 1998; Song et al., 1998), an ATPase which helps to maintain the synaptic expression of GluR2-containing AMPARs (Hanley et al., 2002; Noel et al., 1999). NSF displaces PICK1 from the PICK-GluR2 complex and thereby facilitates the delivery or stabilization of GluR2 at the plasma membrane (Hanley et al., 2002). PICK1 and NSF are also involved in the activity-dependent replacement of GluR2-lacking by GluR2-containing channels at synapses (Gardner et al., 2005; Liu and Cull-Candy, 2005; Lu and Ziff, 2005).

Amyotrophic Lateral Sclerosis (ALS), is a progressive neurodegenerative disorder involving primarily motor neurons of the cerebral cortex, brain stem and spinal cord. While a majority of ALS cases are sporadic, 2-3% of cases are associated with over 100 missense mutations in the enzyme Cu/Zn superoxide dismutase (SOD1) (Cleveland and Rothstein, 2001; Rosen et al.,

1993;Rowland and Shneider, 2001). Recently, in sporadic ALS patients, GluR2 RNA editing in motor neurons was shown to be reduced up to 100%, while the GluR2 editing in Purkinje cells of the same patients was unaltered (Kawahara et al., 2004). This study suggested that failure of GluR2 RNA editing may be one of the underlying causes of sporadic ALS.

AMPA dependent toxicity has not been fully understood, and elucidating its mechanism may aid in devising pharmacologic agents that protect against the toxicity of AMPARs. Here, using a model hippocampal neuron system, we show that the unedited GluR2 forms unusually toxic AMPAR channels and that their toxicity results from GluR2's novel trafficking properties as well as from Ca^{2+} permeability. We show that a peptide that inhibits the constitutive trafficking of unedited GluR2 blocks this toxicity. Limiting trafficking of unedited GluR2 represents a novel pharmacological strategy for blocking excitotoxicity of AMPA receptors.

Results

Excitotoxic death of neurons induced through AMPA receptors

We developed a quantitative TUNEL assay to measure death of cultured dissociated hippocampal neuron resulting from AMPA receptor excitotoxicity. Because this assay was later employed to measure the toxicity of AMPA receptor expressed from sindbis virus (see below), the neurons were infected with a control sindbis virus expressing GFP. Sixteen hours post infection, the neurons were stimulated with various concentrations of AMPA for 15 minutes. The cells were incubated in fresh conditioned media for 4 hours and were processed for the TUNEL assay, which reveals apoptotic cells. Neuron death marked by TUNEL staining increased in a dose dependent manner, with the highest toxicity observed at a 25 μM AMPA stimulus. At a higher, 50 μM stimulus, the cells were grossly disrupted, which likely accounts for the decline in TUNEL positive neurons at this stimulus (Figure 1A). To determine the kinetics of AMPA dependent neuron death, GFP expressing neurons were stimulated with 5 μM AMPA and assayed at various time intervals for TUNEL. AMPA dependent toxicity increased in a time dependent manner starting at 18 h, reaching a maximum at 24h and then declining at 32h (Figure 1B). The decrease in TUNEL positive neurons at 32h most probably reflects disruption of dying neurons. Images of typical neurons at 0h and 24h after AMPA treatment are shown in Figure 1C.

Absence of GluR2 editing at the Q/R site enhances excitotoxicity of AMPA receptors

We next compared the excitotoxic potential of exogenous unedited, GluR2Q subunits with that of edited GluR2R (Figure 2B). Representative images of infected neurons expressing these subunits, either with or without AMPA treatment are shown in Figure 2A. We observed a 2.6 fold increase in TUNEL positive cells following AMPA treatment of edited, GluR2R expressing neurons compared to an unstimulated control, whereas neurons expressing unedited, GluR2Q showed a 7.3 fold increase following AMPA treatment relative to the unstimulated neurons (Figure 2C) and the surface expression of GluR2Q is higher than GluR2R (Figure 2D). These results demonstrated that the GluR2Q is more toxic than edited GluR2 ($P < 0.00001$).

AMPA induced neuron death is a consequence of AMPA receptor activation and Ca^{2+} conductance

We next analyzed the effects of channel mutations and antagonists and of Ca^{2+} chelation on GluR2Q toxicity. The ligand-binding site mutation, R485K, prevents agonist binding (Stern-Bach, 1998), and neurons expressing GluR2Q(R485K) did not show any increase in apoptotic death upon AMPA treatment compared to the untreated control. GluR2Q(R485K) does not traffic to the surface as efficiently as GluR2Q (Greger et al., 2002), however, even after normalizing toxicity for surface expression of the respective subunits, there was no increase

in AMPA dependent death of GluR2Q(R485K) expressing neurons when compared to an untreated control (Figure 3A). Thus neuronal death requires ligand-dependent AMPAR activation. AMPA-induced excitotoxicity in GluR2Q-expressing neurons was completely blocked by CNQX and reduced by nifedipine (Figure 3B), suggesting that excitotoxicity mainly occurs through AMPA receptor activation but that L-type Ca^{2+} channels could play a secondary role. AMPA induced toxicity however was not blocked by APV, suggesting that the excitotoxicity does not involve NMDA receptor activation (Figure 3B). When extracellular Ca^{2+} was chelated by pretreatment of GluR2Q-expressing neurons with BAPTA for 2 minutes, the level of neuron death after AMPA treatment was similar to untreated controls, with or without BAPTA, and was greatly reduced relative to GluR2Q-expressing neurons stimulated with AMPA in the absence of BAPTA (Figure 3C). Thus, GluR2-Q-dependent neuron death required the influx of extracellular Ca^{2+} .

A block to AMPAR desensitization enhances excitotoxicity

Following agonist binding, AMPA receptors desensitize through an agonist binding domain rearrangement that limits channel conductance (Otis et al., 1996). When neurons expressing GluR2Q were treated with cyclothiazide (CTZ), which blocks AMPA receptor desensitization, AMPA treatment induced higher levels of apoptosis compared to control neurons not exposed to CTZ (Figure 3D) and a similar effect was seen for GluR2R expressing neurons. Thus, a block of GluR2Q desensitization imposed by CTZ further enhanced toxicity.

GluR2Q-containing AMPA receptors are more toxic than receptors with other Q-containing subunits

The AMPA receptor subunits GluR1, GluR3 and GluR4 are not edited at the pore apex and contain a glutamine residue at this site. Exogenously expressed GluR subunits largely form homomeric channels (Hayashi and Malinow, 2000; Shi et al., 2001). We asked if stimulation of homomers of other AMPAR subunits with Q at the pore apex, as in GluR1 and GluR3 homomers, more generally leads to toxicity or whether toxicity is a special property of GluR2Q. When the toxicities of the GluR1, GluR2 and GluR3 subunits were compared, GluR2Q showed much higher toxicity upon stimulation with AMPA than to the other Q-containing AMPA receptors (Figure 4A). GluR2Q showed a 5.3 fold increase in TUNEL positive neurons upon AMPA exposure whereas GluR1Q showed 2.8-fold increase in TUNEL positive neurons. Thus GluR2Q exhibits a novel toxicity, even when compared with other Ca^{2+} -permeable AMPA receptors.

AMPA receptors that lack GluR1 but contain GluR2, such as GluR2Q homomers, traffic constitutively to the plasma membrane (Passafaro et al., 2001; Shi et al., 2001). Thus, we asked if the novel high toxicity of GluR2Q reflected an enhanced receptor surface expression relative to other AMPA channel types. The exogenously expressed subunits all contain N-terminal, extracellular epitope tags (Myc tag for GluR2 and GluR3 and HA tag for GluR3). We measured the surface expression of each subunit normalized for the total expression by means of immunocytochemistry (see Experimental Methods) and found that GluR2Q surface expression was higher than the surface expression of GluR1R, GluR1Q, GluR2R and GluR3Q (Figure 4B). These data suggest that the higher surface expression of GluR2Q, which may result from more efficient assembly and rapid trafficking, contributes to its higher toxicity compared to other Q-containing AMPA receptor subunits.

GluR2 C-terminus determines toxicity of 'Q' containing AMPARs

The efficient trafficking and surface expression of GluR2 are facilitated by the specialized chaperone, NSF, which binds to the GluR2 C-terminal domain (Hanley et al., 2002; Nishimune et al., 1998; Osten and Ziff, 1998; Song et al., 1998). We hypothesized that toxicity of GluR2Q, which reflects the capacity for GluR2 trafficking, may also be dependent on NSF. Because the

C-terminal domain of GluR2 binds NSF, we asked if the exchange of the C-terminal domains between GluR1 and GluR2 will change the relative toxicities of these subunits. GluR1Q(R2-CT) (GluR1 with GluR2 C-tail) expressing neurons showed enhanced surface expression and increased toxicity on exposure to AMPA when compared to GluR1Q. GluR2Q(R1-CT) (GluR2Q with GluR1 C-tail) on the contrary showed decreased surface expression and decreased toxicity upon exposure to AMPA when compared to GluR2Q (Figure 5A and 5B). We conclude that the toxicity of GluR2Q is due in part to its enhanced surface expression resulting from functions of the C-tail.

To determine if binding of NSF augments the toxicity of GluR2Q by increasing GluR2Q levels on the neuron surface, we analyzed mutants of GluR2R and GluR2Q that do not bind NSF. We studied a mutant in which the NSF binding site was replaced with the corresponding five amino acids of GluR1 (KVAKN at positions 847-851 of GluR2 is replaced by GFCLIP of GluR1) (Osten and Ziff, 1998). This mutant, GluR2NONSF, does not bind NSF but does bind to other proteins that associate in this region, including AP2 (Lee et al., 2002). GluR2R (NONSF) and GluR2Q(NONSF) surface levels were lower compared to GluR2R and GluR2Q respectively (Figure 6A). The toxicities of GluR2R(NONSF) and GluR2Q(NONSF) were also lowered (Figure 6B), suggesting that the NSF-dependent increases in surface levels of GluR2 also enhance the toxicity of GluR2Q. When toxicity is normalized for surface expression, GluR2R(NONSF) and GluR2Q(NONSF) showed toxicities similar to GluR2R and GluR2Q respectively (Figure 6C). This suggests that the functions of NSF, which are unique to GluR2, enhance GluR2Q toxicity. Images of surface expression of these receptors in neurons are shown in Figure 6D.

To confirm that the function of the GluR2 C tail is dependent on the NSF interaction in the GluR1(GluR2-CT) chimera, we used a chimeric mutant GluR1(GluR2-CT)NONSF, which is incapable of binding NSF. The increase observed in the surface expression of GluR1(GluR2-CT) chimera was abolished in the GluR1(GluR2-CT)NONSF chimera, suggesting that the increase in the GluR1(GluR2-CT) surface expression due to interaction of the NSF (Figure 5B). The toxicity of GluR1(GluR2CT)NONSF upon AMPA stimulation was similar to GluR1 suggesting that the increased TUNEL positivity of Glu1(GluR2-CT) but not GluR1(GluR2-CT)NONSF was increased due to interaction with NSF

Pep2m decreases the toxicity of GluR2Q by decreasing GluR2 surface levels

To block the role of NSF in increasing the surface expression of GluR2Q, we employed a peptide containing the NSF interacting region, pep2m (Noel et al., 1999). In agreement with previous studies (Noel et al., 1999), exposure of neurons to a cell permeant myristoylated form of pep2m decreased the surface levels of endogenous GluR2 and exogenous GluR2R and GluR2Q (Figure 7A). Neurons expressing either GluR2Q or GluR2Q(NONSF) were left untreated, or were treated with pep2m or with a control myristoylated scrambled peptide. Pep2m decreased the toxicity of GluR2Q, while the scrambled peptide failed to change the toxicity. Furthermore, the toxicity of GluR2Q(NONSF) was unaltered by pep2m or by scrambled peptide (Figure 7B). This demonstrates that peptides, and potentially other pharmacologic agents that interfere specifically with GluR2 trafficking, may reduce the toxicity of GluR2Q.

Discussion

Glutamate induced toxicity most commonly involves NMDA receptors and has been implicated in several neurodegenerative diseases (Arundine and Tymianski, 2004). NMDA receptor activation requires coincident pre and postsynaptic activity, due to a voltage dependent block of the channel pore by Mg^{2+} (Coan and Collingridge, 1985; Herron et al., 1986; Mayer et al., 1984). Sustained activation of non-NMDA receptors leads to membrane depolarization

and NMDA receptor activation (Hollmann and Heinemann, 1994). Large influxes of Ca^{2+} through the NMDA receptor lead to uptake of cellular Ca^{2+} by mitochondria resulting in depolarization of the mitochondrial membrane (Schinder et al., 1996). Mitochondrial Ca^{2+} uptake blocks ATP synthesis and the decreased membrane potential inhibits the electron transport chain (Greene and Greenamyre, 1996) resulting in sustained cellular membrane depolarization, leading to further NMDA receptor activation and excitotoxic damage (Greene and Greenamyre, 1996). Mitochondrial Ca^{2+} uptake also promotes release of factors such as cytochrome C and subsequent apoptosis. NMDA receptors also induce nitric oxide (NO) synthesis through the activation of neuronal nitric oxide synthase (nNOS) (Bredt and Snyder, 1989). Under pathological conditions, NO reacts with reactive oxygen species generated by mitochondrial electron transport chain dysfunction to form peroxynitrite, which also kills neurons by nitration of proteins (Dawson and Dawson, 1998).

Unlike the extensive understanding of the toxicity of NMDA receptors, the mechanisms of excitotoxicity of AMPA receptors are not well known. Here we show that GluR2Q forms highly toxic channels and that there are at least two components of this toxicity. The first involves the Ca^{2+} conductance properties of individual channels, and the second, the surface trafficking properties of the channels. In the first category, channel toxicity depends upon the ability of the channel to bind ligand, as expected, and as demonstrated by the reduced toxicity of GluR2Q (R485K), a glutamate non-binding mutant. Furthermore, a block of channel desensitization enhanced toxicity. AMPARs undergo fast desensitization in response to glutamate or AMPA, which renders the receptors in an inactive state while the agonist is still bound, and reduces ion conductance (Sun et al., 2002). AMPA receptor desensitization is blocked by the drug, cyclothiazide (CTZ), which prolongs the opening of the channel, and in this study enhanced toxicity of GluR2Q. Stargazin, an AMPA receptor auxiliary subunit, increases the AMPA receptor response to glutamate by slowing the deactivation rate, by decreasing the rate of desensitization and by accelerating channel recovery to the activated state (Priel et al., 2005; Tomita et al., 2006). Thus stargazin may enhance GluR2 toxicity in the event of an editing loss. The flip alternative splice variant of GluR subunits are more slowly desensitizing than those containing flop variant (Tomiyama et al., 2002). Thus, a change in the ratio of flip to flop variants that alters the desensitization kinetics, may also influence the toxicity.

The trafficking properties of the subunits represent a second channel feature that influences toxicity. A significant proportion of GluR2R is retained in the ER as monomers and dimers because the pore apex arginine imposes a barrier to assembly of multiple GluR2Rs into tetramers (Greger et al., 2003). When RNA editing fails, the GluR2Q that is formed assembles efficiently into functional hetero and homotetramers that overcome the retention barrier and traffic out of the ER. Once the channels are released from the ER, functions of the GluR2 C-terminal domain also contribute to trafficking and as shown here, can contribute to toxicity. Although both GluR1Q and GluR2Q contain Q at the pore apex and assemble readily into tetramers that exit the ER and are Ca^{2+} permeable, GluR2Q containing channels are more toxic because of increased capacity to reach the plasma membrane. When the C-tails of GluR1Q and GluR2R were switched, the trafficking properties of subunits were exchanged, as were their toxicities. The contribution of the C-terminal domain to toxicity was further evaluated by the effects of a NSF binding site mutation. This mutation reduced the trafficking of the receptor to the plasma membrane and also reduced toxicity, as determined for GluR2Q. This indicates that toxicity relies upon the ability of the channels to reach the plasma membrane at high levels and confirms that the trafficking mechanism that underlies toxicity is dependent upon NSF. In cells for which RNA editing has begun to fail, the NSF-dependent mechanism may enable GluR2Q-containing AMPA receptors that are permeable to Ca^{2+} to replace residual Ca^{2+} impermeable GluR1Q/GluR2R heteromeric channels. Such a replacement would enhance AMPAR-dependent Ca^{2+} -currents and could further impair editing and initiate cell death.

Given the apparent role of NSF in toxicity, we asked if disruption of the GluR2-NSF interaction would protect neurons against AMPAR excitotoxicity. We show that pep2m, a peptide mimicking the NSF binding region of GluR2, decreased the toxicity of GluR2Q in hippocampal neurons. These peptide experiments raise the possibility that drugs that block the surface delivery of GluR2Q by interfering with NSF interaction could have therapeutic value following editing failure.

During specific forms of synapse plasticity, Ca^{2+} permeable, GluR1-containing AMPA receptors are replaced by Ca^{2+} impermeable GluR2-containing AMPA receptors. Others (Gardner et al., 2005; Liu and Cull-Candy, 2000) have reported such an AMPAR exchange that is induced by repetitive synaptic activity in cerebellar stellate cells. Neurons may utilize this exchange mechanism to limit Ca^{2+} influx through GluR2-lacking AMPAR. More recently, Plant et al (Plant et al., 2006) have shown that LTP induction in CA1 hippocampal neurons causes a transient incorporation into synapses of GluR2-lacking AMPA receptors and that these receptors are eventually replaced by GluR2-containing AMPA receptors. As with the case of cerebellar stellate neurons, hippocampal neurons may incorporate GluR2-containing AMPA receptors into synapses to limit the Ca^{2+} -conductances of GluR2-lacking AMPAR. If GluR2 editing fails, GluR2Q-containing receptors will be incorporated by the exchange mechanism, defeating the block of the AMPAR-dependent Ca^{2+} currents. In this case, the protective role of GluR2R will be changed by loss of RNA editing to a toxic function following incorporation of GluR2Q.

Perturbations in Ca^{2+} homeostasis may play an important role in the pathogenesis in ALS. In ALS spinal motor neurons are most vulnerable (Van Den Bosch et al., 2000). Significantly, motor neurons do not express Ca^{2+} binding proteins that buffer Ca^{2+} . Such proteins, which are expressed in other neurons, include calbindin, D28K and parvalbumin. In contrast, to spinal motor neurons oculomotor neurons, which are resistant to degeneration during the ALS disease process, express parvalbumin and calbindin (Alexianu et al., 1994; Ince et al., 1993). This suggests that the Ca^{2+} buffering capacity of the neuron may affect its survival. Indeed, motor neurons that overexpress Ca^{2+} binding proteins from a transgene are protected from degeneration in ALS animal models (Ghadge et al., 1997); (Roy et al., 1998). Ca^{2+} homeostasis is deregulated in the motor neurons of transgenic G93A SOD1 mice, which contain vacuoles that are filled with Ca^{2+} , and the cytoplasm and mitochondria are depleted of Ca^{2+} (Siklos et al., 1998). Ca^{2+} permeable AMPA receptors have been implicated in the selective vulnerability of motor neurons (Van Den Bosch et al., 2000). Cultured motor neurons from GluR2-deficient embryos displayed an increased Ca^{2+} influx through AMPA receptors and an increased vulnerability to AMPA receptor-mediated excitotoxicity (Van Damme et al., 2005). Expression in motor neurons of edited GluR2 reduced two factors that contribute to the death of the SOD^{G93A} mice, Ca^{2+} permeability of AMPA receptors and SOD misfolding (Tateno et al., 2004). Significantly, elevated levels of wild type GluR2R expressed from a transgene in motor neurons overcame the toxicity of the G93A SOD mutation. This suggests that Ca^{2+} fluxes through AMPAR (presumably through GluR2-lacking channels since the editing is normal in these mice) can contribute to the toxicity of the mutant SOD and that these fluxes can be suppressed by elevating the levels of Ca^{2+} -impermeable channels. Because neurodegeneration in this model apparently involves a Ca^{2+} -dependent enhancement of SOD misfolding, such misfolding could increase if editing fails. It will be interesting to determine if Ca^{2+} buffering proteins and Ca^{2+} -impermeable AMPAR protect neurons by limiting glutamate induced, Ca^{2+} mediated SOD misfolding in familial form of the disease.

Animals with a deficiency in GluR2 Q/R site editing die prematurely as a consequence of neuronal death (Brusa and Seeburg, 1995) and mice transgenic for a pore site mutation that increases Ca^{2+} -permeability, GluR2N, develop late onset motor neuron disease (Kuner et al., 2005a). These results support the conclusion that a reduction of editing efficiency at the GluR2

Q/R site or other changes that increase Ca^{2+} -permeability are possible contributors to neuronal death (Kuner et al., 2005a;Kuner et al., 2005b).

Failure of RNA editing of GluR2 at the Q/R site may lead to death of motor neurons during sporadic ALS (Kawahara et al., 2004;Takuma, 1999). The editing mechanism itself in these neurons may be impaired, since editing of GluR2 in rodents has been reported to be nearly complete in all regions of the brain under physiologic conditions (Kamphuis and Lopes da Silva, 1995;Kortenbruck, 2001). Under pathological circumstances, the extent of editing of GluR2 at the pore apex may be regulated by changes in the level of ADAR2 mRNA or protein, since a lower level of ADAR2 mRNA has been correlated with lower editing in human brain white matter (Kawahara et al., 2003) Other factors such as alternative splicing and translation of ADAR2 mRNA may also determine the extent of GluR2 mRNA editing (Akbarian et al., 1995;Nutt and Kamboj, 1994).

In the case of ALS patients, reduced RNA editing at the GluR2 Q/R site is observed in individual motor neurons, with the degree of editing loss varying from 100-0%. Impaired GluR2 editing is also seen in other diseases, such as in malignant gliomas, where the self-editing and splicing of ADAR2, rather than ADAR2 levels, are affected (Maas et al., 2001). This implies that RNA editing at the GluR2 Q/R site may have a role in tumor progression. Similarly, reduction in GluR2 editing at the Q/R site in human oligodendrocytes may be a pathogenic factor in multiple sclerosis, in which AMPAR mediated neuronal death has been implicated (McDonald et al., 1998;Pitt et al., 2000;Smith et al., 2000).

As discussed earlier, Ca^{2+} can be toxic to the cell. In the case of the toxicity of Ca^{2+} conducted by NMDAR, the requirement for coincident activity for NMDAR activation provides a safeguard. In contrast, there is no requirement for coincident activity for activation of the AMPAR. Thus, another mechanism must be provided to guard against this Ca^{2+} toxicity. Neurons express both Ca^{2+} permeable and Ca^{2+} impermeable AMPAR (Tanaka et al., 2000). Significantly, the Ca^{2+} permeable channels, which would correspond primarily to GluR1 homomers, require activity for their trafficking to the synapse (Shi et al., 2001). The requirement for activity for trafficking limits the conditions under which Ca^{2+} permeable AMPA currents can be generated. An additional mechanism is also utilized for limiting Ca^{2+} permeable AMPAR currents. Ca^{2+} fluxes and tetanic stimulation can induce the endocytosis of Ca^{2+} permeable AMPAR in stellate neurons, and these channels are replaced by Ca^{2+} impermeable channels, ones that contain GluR2 (Gardner et al., 2005;Liu and Cull-Candy, 2000). This mechanism of replacement utilizes the PICK1 protein and NSF. When cells utilize the rapid trafficking of Ca^{2+} impermeable AMPAR to the synapse to replace Ca^{2+} permeable channels, these Ca^{2+} impermeable channels do not pose a threat of Ca^{2+} toxicity. This mechanism for reducing AMPAR-dependent Ca^{2+} currents relies on the near quantitative editing of GluR2, which indeed has been observed under most physiological circumstances. If editing fails, GluR2 subunits are formed that retain the capacity for rapid, activity-independent trafficking to synapses, but are incapable of limiting Ca^{2+} entry. Thus, when editing fails, the mechanism used by the cell to limit AMPAR-dependent Ca^{2+} entry also fails and the cell may be exposed to continued excessive Ca^{2+} currents, leading to toxic death.

Figure 8 compares the ion conductances of AMPAR types under different conditions of activity RNA editing and pharmacologic treatment. GluR1 channels, although Ca^{2+} permeable, require activity for their incorporation in to the synapse. GluR2 and GluR3-containing channels are constitutively trafficked to the surface but are Ca^{2+} impermeable. Only unedited GluR2, acting either as a homomer or as a heteromer with GluR3, is both Ca^{2+} permeable and efficiently trafficked to the surface, with surface expression dependent on NSF. Using a peptide to block interaction of NSF with unedited GluR2 reduces the Ca^{2+} currents and the toxicity.

We have shown that the delivery of the peptide pep2m, and potentially other small molecules that prevent the GluR2Q-NSF interaction can limit the delivery of GluR2Q to the neuron surface and reduce toxicity. Further experiments employing pep2m to limit GluR2Q toxicity *in vivo* in animals will prove useful in substantiating a therapeutic value for pep2m. Other small molecules such as peptide mimetics that interfere with the trafficking of unedited GluR2Q may also be used to lower toxicity.

Experimental Methods

Primary neuronal cultures

Neurons were obtained from E19 Sprague Dawley rat embryos and were maintained and processed as described (Osten et al 1998). For TUNEL assay and immunostaining, hippocampi were dissected, dissociated and cells were plated at 150,000 cells per well on coverslips precoated with poly-L-Lysine in a 6-well plate.

Attenuated Sindbis virus construction

Recombinant attenuated Sindbis viruses were constructed by subcloning a new multiple cloning site into the attenuated Sindbis vector and then subcloning the cDNAs of GluR1, GluR2 and their mutants, as described previously (Greger et al., 2003; Osten et al., 2000), into the modified attenuated Sindbis vector. All GluR2 and GluR3 subunits and their mutants are Myc epitope tagged at the extracellular N-terminus. The GluR1 subunit and its mutants are HA epitope tagged at the N-terminus.

Infection and TUNEL assay

Neurons 14 DIV on cover slips were infected with attenuated Sindbis virus expressing tagged GluR subunits for 16-17 hours and then treated with 5 μ M AMPA for 15 min. Cells were incubated for 7-8 hours or as indicated, post treatment and then were fixed in 4% paraformaldehyde and permeabilized using 0.2% Triton-X-100. TUNEL assay was performed as per instructions using a kit from Roche (Nutley NJ). Exogenous receptor expression was detected by immunocytochemistry using epitope specific antibodies, anti-Myc or anti- HA depending on the subunit epitope tag. Fluorescent-tagged secondary antibodies were used for detection. Images were captured on a Nikon PCM 2000 confocal microscope, and analyzed using simple PCI imaging software (C-Imaging, Mars, PA).

Quantification of the surface expression

Hippocampal neurons 14 DIV on coverslips were infected with Sindbis virus expressing GluR2, GluR3 or GluR1, each with an epitope tag in the extracellular N-terminal domain. Tags were Myc for GluR2 and GluR3 and HA for GluR1. Sixteen hours post-infection neurons were live stained for GluR subunits on the cell surface with 4 μ g/ml of primary antibodies (anti Myc Santa Cruz Biotechnology, Santa Cruz CA., and anti-HA Covance, Berkeley, CA.) raised in rabbit, for 10 min at 37° C with 5% CO₂. Neurons were then fixed in 4% paraformalehyde and permeabilized with 0.2% Triton X-100, to permit staining of internal GluR subunits. Total expression was detected with mouse primary anti-Myc or anti-HA antibodies. The surface and internal receptors were detected by anti rabbit FITC and anti mouse rhodamine conjugated secondary antibodies as appropriate. To quantify expression of GluR2 and GluR1, cell areas were defined as the extent of total Myc and HA surface staining, and total intensity was measured in this area. The ratio of surface signal to cell area was calculated.

Treatment with peptides

Hippocampal neurons were infected with attenuated sindbis viruses expressing GluR2Q or GluR2Q(NONSF). Seventeen hours post infection the samples were washed with artificial

cerebro spinal fluid (ACSF, containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 10 mM glucose) and incubated with 50 μM of pep2m (myr-KRMKVAKNAQ) (Pepsyn, Liverpool, UK), or scrambled peptide (SCR) for 45 min in ACSF. When samples were AMPA treated, AMPA was added during the last 15 min of the peptide incubation. Surface expression of GluR2Q and GluR2Q(NONSF) was measured and TUNEL assay was performed both as described above.

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Figure 1

Figure 1. Kinetics of AMPA mediated excitotoxicity involving endogenous receptors

A) Dose dependent AMPA mediated excitotoxicity. GFP expressing neurons were exposed to AMPA as indicated for 15 min. Neurons were fixed, assayed for TUNEL and immunostained after 4 hours. TUNEL positive neurons were quantified using confocal imaging and Simple PCI software. B) Hippocampal neurons (14DIV) infected with sindbis virus expressing GFP were exposed to 5 μ M AMPA for 15 min and fixed for immunocytochemistry at the times indicated. Infected neurons were identified in cultures by immunostaining with anti-GFP antibody. Neurons were imaged using confocal microscopy, and TUNEL positive neurons were quantified by Simple PCI software. C) Images of 14 DIV hippocampal neurons treated with AMPA for 0 and 24 h immunostained with anti-GFP antibody and TUNEL are shown.

Experiments in panel A have been repeated 3 times and the experiments represented in panel B have been carried out four times. Ten images from each sample have been used for quantification for all experiments.

Figure 2

Figure 2. GluR2Q shows enhanced toxicity upon AMPA treatment

A) Hippocampal neurons at 14 DIV were infected with sindbis virus expressing either GluR2R, or GluR2Q. These subunits have an N-terminal, extracellular Myc tag. Sixteen hours post infection the samples were either untreated for controls or treated with 5 μ M AMPA for 15 minutes and then incubated for 8 h. The samples were immunoassayed with anti-Myc antibody and then assayed for TUNEL. Images captured on a confocal microscope are shown. P value of R2 versus R2Q is 0.000007. B) GluR2 subunit structure showing the pore loop Q/R site. C) Quantification of TUNEL positive neurons (shown in Figure 2A) expressing GluR2R or GluR2Q with and without AMPA treatment. value of <0.05=*, <0.005=**, <0.0005=***. D) Surface expression of GluR2R and GluR2Q is presented as the ratio of surface expression to total expression of GluR2. The images of in panel A are representative of 9 experiments, and ten images were used from each experiment for quantification in panel C. A=AMPA.

Figure 3

Figure 3. Dependence of AMPA induced neuronal death on receptor function

A) Hippocampal neurons 14 DIV were infected with sindbis viruses expressing either GluR2 or the agonist non-binding mutant, GluR2Q(R485K). Sixteen hrs post infection the samples were either left untreated (control) or treated with 5 μ M AMPA for 15 min. (P value of R2Q +A versus R2Q(R485K)+A is < 0.011). B) Hippocampal neurons 14DIV were infected with sindbis virus expressing GluR2Q. The neurons were treated with either AMPA or with AMPA plus the AMPA receptor antagonist, CNQX (20 μ M), or with AMPA plus 100 μ M APV, a NMDA receptor antagonist, AMPA plus Nifedipine (2 μ M), a L-type Ca²⁺ channel blocker. Neurons were immunoassayed for subunit expression using Myc antibody and assayed for TUNEL. P value of R2Q +A versus R2Q+A+CNQX is 0.002. P value of R2Q+NIF versus

R2Q+NIF+A is 0.04 and the P value for R2Q+APV versus R2Q+APV+A is 0.05. C) Hippocampal neurons 14 DIV expressing GluR2Q were left untreated (control) or treated with AMPA or with AMPA plus the extracellular Ca^{2+} chelator, BAPTA, or with BAPTA alone (P value of R2Q+A versus R2Q+A+BAPTA is 0.005). D) Hippocampal neurons expressing GluR2Q were either treated or not with AMPA, AMPA plus cyclothiazide, a desensitization blocker, or cyclothiazide alone. All neurons (A-D) were immunostained with anti-Myc antibody and assayed for TUNEL (P value of R2Q+ versus R2Q+A+CTZ is 0.022). The images were captured using a confocal microscope and quantified. P value of $<0.05=*$, $<0.005=**$, $<0.0005=***$. The data represented in Figure 3 are averages of 3 experiments each. A=AMPA, NIF=nifedipine, CTZ= cyclothiazide.

Figure 4

Figure 4. GluR2Q is more toxic compared to GluR1 and GluR3

A) Hippocampal neurons 14 DIV were infected with sindbis virus expressing GluR1R, GluR1Q, GluR2R, GluR2Q or GluR3Q. The neurons were either untreated (control) or treated with 5 μ M AMPA for 15 min. Neurons were immunostained with either anti HA or anti Myc antibody for measuring receptor surface expression as described in Experimental Methods and assayed for TUNEL. The images were captured on a confocal microscope and quantified. The P value of R1Q+A versus R2Q+A is 0.0045. B) Surface expression of the subunits tested in Figure 4A was quantified as described in the Methods section using PCI software. P value of <0.05=*, <0.005=**, <0.0005=***. The data shown in panels A and B are averages of 3 experiments each. A=AMPA.

Figure 5

Figure 5. Relative toxicities of chimeric subunits

A) Hippocampal neurons 14 DIV were infected with Sindbis virus expressing the indicated subunits, and were either unstimulated or stimulated with 5 μ M AMPA for 15 min. Neurons were immunostained with either anti HA for GluR1 subunits or anti Myc for GluR3 subunits antibody while living to detect surface receptor or permeabilized and then assayed for TUNEL. TUNEL positive neurons were quantified using confocal images. P value between R1Q(R2CT)+A and R2Q(R1CT)+A is 0.039. B) Comparison of the surface levels of subunits in Figure 5A, quantified as described in Experimental Methods. P value of R1Q versus R1Q(R2-CT) is 0.029, R2Q versus R2Q(R1-CT) is 0.005. P value of $<0.05 = *$, $<0.005 = **$,

<0.0005=***. The data shown in panels A and B are the averages of 3 independent experiments each. A=AMPA.

Figure 6

Figure 6. Toxicity of GluR2Q in part depends on its ability to bind NSF

A) Surface expression of the GluR2R, GluR2R(NONSF), GluR2Q and GluR2Q(NONSF) were determined by immunostaining living cells for surface and total levels and quantified using simple PCI software. P value of GluR2Q versus GluR2Q(NONSF) is 0.003. B) Hippocampal neuron 14 DIV were infected with sindbis virus expressing either GluR2R, GluR2Q, or NSF binding mutants, GluR2R(NONSF) or GluR2Q(NONSF). The neurons were either unstimulated or stimulated using 5 μ M AMPA for 15 min. The neurons were immunostained with anti Myc antibody and assayed for TUNEL. P value of R1Q versus R1Q(R2-CT) is 0.03 and GluR2Q+A versus GluR2Q(NONSF)+A is .0086. C) TUNEL positive neurons shown in Figure B are normalized to surface expression. D) Images of neurons for used for quantification of surface to total ratios. P value of <0.05= *, <0.005=**, <0.0005=***. The experiments shown in this Figure were repeated 4 times. A=AMPA

Figure 7

Figure 7. NSF interacting peptide pep2m reduces toxicity of GluR2Q

A) Neurons expressing GluR2Q and GluR2Q(NONSF) were treated either with pep2m (myr-KRMKVAKNAQ) or scrambled peptide (SCR) and control neurons without peptide treatment were assayed for surface expression as described in the methods. P value of GluR2Q surface expression versus GluR2Q+ pep2m is 0.005. B) Neurons were infected with sindbis virus expressing the indicated subunits and treated with pep2m or scrambled peptide for 1hr prior to AMPA (5 μ M for 15 minutes) treatment. Post treatment neurons were immunoassayed with anti-Myc antibody and then assayed for TUNEL. Images of TUNEL positive neurons expressing GluR2Q, GluR2Q+pep2m, GluR2Q+SCR, GluR2Q(NONSF), GluR2Q(NONSF)+pep2m, GluR2Q(NONSF)+SCR were captured on a confocal microscope and quantified. P

value between GluR2Q+A and GluR2Q+A+pep2m is 0.027. P value of $<0.05=*$, $<0.005=**$, $<0.0005***$. The data shown in this Figure represent the averages of 3 independent experiments. A=AMPA.

Figure 8. Factors influencing AMPAR plasma membrane insertion and resulting Ca^{2+} currents
 Model showing effects of AMPAR Ca^{2+} permeability and surface trafficking on AMPAR Ca^{2+} currents. Increased Ca^{2+} currents are related to toxicity. a) Receptors containing only GluR1 are Ca^{2+} permeable but these receptors require activity for plasma membrane insertion, and hence Ca^{2+} currents are limited. b) AMPAR containing GluR2/3 insert into the plasma membrane by an activity-independent and NSF-dependent mechanism, but since they are Ca^{2+} impermeable, Ca^{2+} currents are limited. c) AMPAR containing unedited GluR2 and GluR3 (GluR2Q/3) insert into the plasma membrane by an activity-independent, NSF-dependent mechanism and are Ca^{2+} permeable, leading to large Ca^{2+} currents. d) Blocking the action of NSF with a peptide reduces the plasma membrane insertion of GluR2Q/3 channels, reducing Ca^{2+} currents.