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NEUROPROTECTION BY GLUTAMATE RECEPTOR ANTAGONISTS AGAINST SEIZURE-INDUCED EXCITOTOXIC CELL DEATH IN THE AGING BRAIN

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Abstract

We previously have identified phenotypic differences in susceptibility to hippocampal seizure-induced cell death among two inbred strains of mice. We have also reported that the age-related increased susceptibility to the neurotoxic effects of seizure-induced injury is regulated in a strain-dependent manner. In the present study, we wanted to begin to determine the pharmacological mechanism that contributes to variability in the response to the neurotoxic effects of kainate. Thus, we compared the effects of the NMDA receptor antagonist, MK-801 and of the AMPA receptor antagonist NBQX on hippocampal damage in the kainate model of seizure-induced excitotoxic cell death in young, middle-aged, and aged C57BL/6 and FVB/N mice, when given 90 minutes following kainate-induced status epilepticus. Following kainate injections, mice were scored for seizure activity and brains from mice in each age and antagonist group were processed for light microscopic histopathologic evaluation seven days following kainate administration to evaluate the severity of seizure-induced injury. Administration of MK-801 significantly reduced the extent of hippocampal damage in young, mature and aged FVB/N mice, while application of NBQX was only effective at attenuating cell death in young and aged mice throughout all hippocampal subfields. Our results suggest that both NMDA and non-NMDA receptors are involved in kainate-induced cell death in the mouse and suggest that aging may differentially affect the ability of neuroprotectants to protect against hippocampal damage. Differences in the effectiveness of these two antagonists could result from differential regulation of glutamatergic neurotransmitter systems or ion channel specificity.

Keywords

kainic acid; MK-801; NBQX; neuronal damage; hippocampus; mouse strain

Introduction

Glutamate is the major excitatory neurotransmitter in the CNS. Through the activation of N-methyl-D-aspartate (NMDA) and non-NMDA receptors, it is involved in important neurophysiological functions such as neurodevelopment, neuroproliferation, and learning and memory (Daoudal and Debanne, 2003; Frebel and Wiese, 2006). However, excessive

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activation of the NMDA and non-NMDA subtype of glutamate receptors leads to excitotoxic neuronal death (Choi, 1988; Choi and Rothman, 1990). Glutamate-induced neurotoxicity has been shown to play a critical role in the neuronal damage and death underlying a wide range of central nervous system disorders, including ischemic stroke (reviewed in Hazell, 2007; Bessancon et al., 2008), epilepsy (Parsons et al., 1998; Krystal et al., 1999), as well as numerous neurodegenerative diseases (Lipton, 2004; Johnston, 2005; Fan and Raymond, 2007).

The cellular and molecular events responsible for selective vulnerability of neurons to excitotoxic damage have been the subject of much speculation. It has been hypothesized that a variety of cascades involving biochemical and electrophysiological events combine to produce neuronal damage (Choi, 1992; Whetsell, 1996; Michaelis, 1998; Nicholls and Budd, 1998; Sattler and Tymianski, 2000). One leading hypothesis purports that activation of ionotropic glutamate receptors by high levels of glutamate can trigger dysregulated homeostasis of calcium and other cellular ions (Bano and Nicotera, 2007), resulting in influx of Ca^{2+} and Na^{+} into the damaged cells (Bullock, 1993; Luer et al., 1996). Accumulation of intracellular Ca^{2+} is accompanied by marked degradation of membrane glycerophospholipids, the accumulation of lipid peroxides (Farooqui and Horrocks, 1994), and the initiation of signaling cascades within susceptible neurons resulting in cell death.

Insight into potential contributory factors that may play a role in vulnerability to excitotoxic cell death has arisen from studies examining the molecular aspects of glutamate receptors. Each of the ionotropic receptor types is composed of at least four subunits that come together to form a functional ion channel and ion permeability is determined, in part, by the subunits that form each receptor type. The differential distribution of glutamate receptor subtypes in the brain, as well as the diverse composition of the subunits suggests differences in the pathophysiological outcome of excitotoxicity, as related subunits can be combined to form glutamate receptors with different pharmacological and physiological properties (Hollmann et al., 1991; Hume et al., 1991). Considering the seemingly pivotal role of Ca^{2+} in glutamate receptor-mediated excitotoxicity, it is clear that the precise receptor subunit composition may be crucial in determining the vulnerability of neurons to excitotoxic insults.

Kainic acid (KA) is an exogenous glutamate analogue frequently used as a tool to experimentally mimic human temporal lobe epilepsy. Among the most classical models of human temporal lobe epilepsy, it has long been known that systemic or intracerebral injection of kainic acid in rodents induces epileptiform seizures (Nadler, 1981; Olney, 1981; Ben-Ari, 1985; reviewed in Ben-Ari and Cossart, 2000). It is thought that seizures originate in the CA3 region of the hippocampus, spread to other limbic structures and can be followed by neuronal loss in selected regions of the brain, similar to brain damage seen in patients with temporal lobe epilepsy (Sperk et al., 1983; Ben-Ari, 1985; Sloviter, 1994; Loscher, 1997; Mathern et al., 1997; Zhang et al., 2002). Hippocampal sclerosis most frequently involves extensive loss of cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) pyramidal cells and mossy cells of the dentate hilus, with relative sparing of dentate granule cells (Nadler et al., 1978; Ben-Ari, 1985). It is generally thought that this cell death is linked to pathologically increased release of the excitatory neurotransmitter glutamate, as administration of glutamate antagonists, especially those that block the N-methyl-D-aspartate (NMDA) receptor, are neuroprotective under these conditions (Clifford et al., 1990; Sperk, 1994; Lancelot and Beal, 1998).

Previously, we had reported that age-related increased susceptibility to the neurotoxic effects of seizure induction and seizure-induced injury by systemic kainate administration is regulated in a strain-dependent manner (McCord et al., 2008), similar to previous observations in young adult mice (Schauwecker and Steward, 1997). In particular, resistance to seizure-induced excitotoxic cell death continues throughout the lifespan of C57BL/6J mice, and secondly, aging increases the behavioral seizure sensitivity to the pro-convulsant actions of systemic kainate

administration irrespective of the mouse strain. As well, vulnerability to seizure-induced neuropathology in FVB/NJ mice (excitotoxic cell death susceptible) is exacerbated in aged mice (McCord et al., 2008). In particular, aged animals appear to exhibit greater excitotoxic damage following experimental kainate than young controls (Auer, 1991; Wozniak et al., 1991; Kesslak et al., 1995). At present, the mechanisms responsible for age-dependent regulation of seizure-induced cell damage remain unclear and few studies have rigorously examined the specific mechanisms underlying the supersensitivity to excitotoxic damage seen in aged animals. As the process of excitotoxic neuronal death elicited by KA administration can be influenced by differential activation of glutamate receptor subtypes, we wanted to determine whether glutamate receptor antagonists acting at either the AMPA or NMDA receptor are as effective in aged animals as they are in young animals, with respect to their ability to protect against seizure-induced cell loss. Specifically, we have determined in the present study whether the neurotoxic effects of kainate administration can be prevented by administration of the NMDA antagonist, MK-801, or the AMPA receptor antagonist, NBQX.

Materials and Methods

Animals

Young (2-month-old), middle-aged (12 months) and old (18-months) male C57BL/6J (B6) mice were obtained from the NIA aging colony. FVB/NJ (FVB) mice were purchased from Taconic (Tarrytown, New York) at 2 and 7–8 months of age and then aged in-house for an additional period of 5–11 months before use. Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Southern California. Every effort was made to minimize the number of animals used and their suffering.

Kainate-induced status epilepticus (KA-induced SE)

Kainic acid (KA; Diagnostic Chemicals, Ltd., Charlottetown, PEI, Canada) was dissolved in isotonic saline (pH 7.3) and administered s.c. to adult male mice. In a prior study (McCord et al., 2008), we had conducted dose response testing in 3–4 groups of male mice from each strain. Preliminary dose response studies had defined seizure thresholds and revealed consistent Stage 5 seizures (ED₈₀; effective dose at which 80% of the mice exhibited Stage 5 seizures) and a mortality of less than 30% among both young adult mouse strains at a dose of 25 mg/kg, s.c., among mature mice at a dose of 20 mg/kg, s.c., and among aged mice at a dose of 15 mg/kg, s.c. (McCord et al., 2008). These doses ensured stage 5 seizures and minimized mortality, and also minimized the probability for any aging-related differences in latency to convulsive seizures as well as their duration. KA solutions were prepared fresh on the day of each experiment and each animal received only one injection.

Seizure testing

Following KA injections, mice were placed in clear plastic cages and monitored every 15 min for 4 h for the onset of locomotor activity and behavioral manifestations of limbic seizure episodes. As described previously (McCord et al., 2008), irrespective of age, these episodes commenced with automatisms including staring, rigidity, and immobility, followed by jaw movements, blinking and head bobbing, and forelimb clonus. The next stage of seizures which consisted of rearing, forelimb/head clonus, and tonic/clonic seizures, postural imbalance, and uncontrolled running and jumping defined the latency to first maximal seizure. Status epilepticus (S.E.) was defined as continuous behavioral seizure activity lasting at least 1 h or a series of intermittent seizures without restoration of normal behavioral patterns between successive seizures.

Mice were scored for seizure activity using a previously established six point seizure scoring scale (Schauwecker and Steward, 1997) that was adapted from a five-point scale for rats (Racine, 1972). Seizure stages were defined as follows: stage 1, immobility; stage 2, forelimb and/or tail extension, rigid posture; stage 3, repetitive movements, head bobbing; stage 4, rearing and falling; stage 5, continuous rearing and falling; stage 6, severe tonic-clonic seizures. Seizure parameters monitored included latency of convulsions, duration of seizure activity, and mortality.

Antagonist administration

We evaluated the effectiveness of two anticonvulsants, MK-801 and NBQX, to prevent status epilepticus-induced limbic brain damage. (+)-MK801 maleate salt [dizocilpine; (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] and NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f) quinoxaline-7-sulfonamide disodium) were obtained from Sigma Aldrich (St. Louis, MO). Ninety minutes following kainate-induced status epilepticus, each group was treated with i.p. injections of one of the two antagonists at two different doses (MK-801: 0.5 or 1.0 mg/kg; NBQX: 30 or 60 mg/kg). We chose to administer glutamate receptor antagonists after induction of status epilepticus as administration before induction interferes with seizure activity (Bertram and Lothman, 1990) and limbic epileptogenesis (Rice and DeLorenzo, 1998). A control group received only an i.p. injection of a similar volume of saline solution 90 minutes following kainate administration.

All compounds were administered i.p. as a freshly prepared solution in sterile saline (0.9% NaCl) and administered in a volume of 0.1 ml/10g of mouse body weight. When necessary, pH was adjusted to 7.3–7.4 by adding 0.2N HCl. Doses of the two antagonists were chosen based on their proven effectiveness in preventing epileptic manifestations in experimental models of seizures (Rubaj et al., 2003; Ferreri et al., 2004).

Tissue preparation and histological staining

In order to evaluate the severity of brain damage associated with seizures at different ages, brains from animals in each group were processed for light microscopic histopathologic evaluation according to previously published methods (Schauwecker, 2000; Schauwecker et al., 2000; Schauwecker, 2002a,b; Santos and Schauwecker, 2003; Schauwecker et al., 2004). Briefly, seven days following kainate administration, mice were deeply anesthetized with Avertin and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed and post-fixed overnight, and left in 30% sucrose for at least 12–18 hours for cryoprotection. Horizontal (40 μ m) frozen sections were cut on a sliding microtome and immersed in 0.1M phosphate buffer (pH 7.4), free-floating until histological processing was begun.

Every sixth section (~240 μ m) was processed for cresyl violet staining to assess cell loss and morphology. An alternate series of sections (~240 μ m) were stained with a modification of the selective Fink-Heimer silver stain, which stains degenerating fibers, synaptic terminals, and cell bodies (Nadler and Evenson, 1983), and examined for the appearance of degenerative debris. Briefly, sections were washed in distilled water, pretreated in a solution containing 4.5% sodium hydroxide and 0.06% ammonium nitrate for 5 min, and then incubated for 10 min in an impregnating solution containing 5.4% sodium hydroxide, 6.4% ammonium nitrate, and 0.3% silver nitrate. Sections were then washed three times for a total of 5 min in a solution containing 28.5% ethanol, 1.2% ammonium nitrate, and 0.5% sodium bicarbonate, and then transferred to a developing solution containing 0.5% citric acid, 5% formalin, 9.5% ethanol, and 1.2% ammonium nitrate. Sections were rinsed briefly, mounted onto gelatinized slides, dehydrated through graded alcohols, cleared in xylene and coverslipped.

NeuN immunofluorescence

Immunofluorescence was performed on an additional series of sections (every sixth section; ~240 μm) to detect those neurons that survived seven days following kainate-induced SE. Sections were incubated in 0.1M phosphate buffer (pH 7.4) containing 5% normal serum and 0.1% Triton X-100. Next, sections were incubated with a neuronal marker against NeuN (monoclonal from mouse; Chemicon; 1:500) for 12 h at 4°C. A secondary antibody from mouse (CY2 to detect NeuN) 1:200 was applied for 2 h. After rinsing, sections were mounted and coverslipped with ProLong anti-fade mounting medium (Molecular Probes, Eugene, OR). For labeling, omission of the primary antibody served as a negative control. Labeling for NeuN was viewed under an Olympus BX51 fluorescence microscope (Olympus, New York).

Neuronal loss quantification

We counted cells in defined areas of CA1, CA3, the dentate hilus, and the dentate gyrus in a blinded manner on Cresyl Violet-stained sections as described (Schauwecker and Steward, 1997; Schauwecker et al., 2000). The number of Nissl-stained neurons in area CA3, area CA1, the dentate hilus, and the dentate gyrus was counted in both the right and left hippocampus. Hippocampal subfields were based on Franklin and Paxinos (1997) classification and discrimination between the CA3 and dentate hilus region was based on morphological features and locations of the cells (West et al., 1991). Specifically, for dentate hilar cell counts, the hilus was operationally defined as the region bordered by the supra- and infrapyramidal granule cell layers and excluding the densely packed pyramidal neurons of area CA3.

Neuron counts were made in all subfields and in both hemispheres of each mouse. Values for each side were averaged into single values for each animal. Surviving cells were counted only if they were contained within the pyramidal cell layer or dentate hilus, possessed a visible nucleus and characteristic neuronal morphology, and had a cell body larger than 10 μm . Counting was initiated within the ventral hippocampus at the first point where hippocampal subfields could be easily identified. This level corresponded to horizontal section 54, based on the atlas of Sidman et al. (1971). Six square counting frames (200 $\times$ 200 μm) were randomly placed in the pyramidal layer of fields CA1 and CA3 or in the dentate hilus in four to five regularly-spaced horizontal sections from each animal that were at least 240 μm apart. Only those neuronal nuclei in the focal plane were counted with a 40X objective and considered as a counting unit. Neuronal counting was performed with the aid of Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD, USA) in combination with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a motorized Z-stage (Optiscan, Prior Scientific, Fairfax, VA). All data were expressed as average number of neurons per field and final cell counts are expressed as the percentage of cells as compared with intact mice.

Statistical analysis

Data were presented as mean $\pm$ SEM, and differences between groups were compared statistically by one-way analysis of variance using the computer program, SigmaStat (Jandel Scientific, San Rafael, CA). Intergroup differences were analyzed by the Student-Newman-Keuls post hoc test. Data were considered to be statistically significant when the probability value was <0.05 .

Results

Induction of status epilepticus by systemic kainate

Following systemic administration of KA, mice of all ages ($n=132$), underwent a series of behavioral seizure stages as previously described (Schauwecker et al., 2000, 2004; McCord et al., 2008). Within 10 min of injection, mice assumed a catatonic phase and were immobile.

Within 20–30 min post-injection, mice exhibited forelimb clonus and hindlimb clonus, and within 45 min post-injection, nearly all mice were rearing and exhibited tonic-clonic seizures. Seizures lasted an average of 61.4 ± 1.19 min in young adult mice and no significant strain-dependent effects were found with regard to the latency or duration of seizures in this age group. Similarly, seizures lasted an average of 55.05 ± 0.78 min in mature mice and an average of 53.5 ± 0.85 min in aged mice. No significant strain-dependent effects were found with regard to the latency or duration of seizures in either the mature or aged groups, as well. These results are identical to our previous results demonstrating no strain differences in seizure parameters among young adult, middle-aged or aged mice (McCord et al., 2008). As well, no qualitative differences in seizure intensity were observed between the two strains irrespective of the age of the animal or the antagonist administered 90 minutes following kainate.

Morphologic changes after KA without drug treatment

As we have reported previously (Schauwecker et al., 2000; 2004), the systemic administration of kainic acid induced significant morphologic changes with massive loss of the principal cells in Ammon's horn and hilus. Consistent with previous studies in rats (Nadler et al., 1980a; Sperk et al., 1983; Ben-Ari, 1985) and in mice (Schauwecker et al., 2000, 2004; McCord et al., 2008), administration of KA led to the degeneration and loss of CA3 pyramidal cells and hilar neurons, and sporadic reduction of neurons in CA1 pyramidal cells in FVB (susceptible) mice. Increased argyrophilic deposits were present throughout the stratum oriens and stratum pyramidale of the CA3 and CA1 subfields and within the dentate hilus. In accordance with previous studies (Nadler et al., 1980a,b; Sperk et al., 1983), cells within the dentate granule cell layer and area CA2 of Ammon's horn were resistant to kainate-induced cell damage in susceptible mice. In contrast, B6 mice exhibited no detectable reduction of neurons within the hippocampus proper, and no indication was noted of damage to neuronal nuclei in any hippocampal region or in the septum, amygdala, pyriform cortex, neocortex, or thalamic nuclei in cresyl violet-stained sections, irrespective of the age of the mice. In addition, no Fluoro-Jade fluorescence staining or signs of degeneration, as assessed using selective silver stains, were noted in B6 animals that received KA.

Quantitative analysis of subfield group means revealed that mice susceptible to Kainate-induced cell death (FVB) displayed a reduction of 66–95% of dentate hilar neurons, depending on the age of the animal, 61–81% of CA3 pyramidal neurons, depending on the age of the animal, and 45–72% of CA1 pyramidal neurons, depending on the age of the animal, as evidenced by decreased cresyl violet and NeuN-immunostaining (see Fig. 4) and increased Fluoro-Jade staining. In contrast, resistant mice (B6) displayed no detectable evidence of degenerative debris or reduction in neurons in any of the hippocampal subfields after KA administration, irrespective of age. These results are confirmatory of our previous study reporting an age-related increased vulnerability of the hippocampus to seizure-induced cell death in FVB mice and a maintained resistance to seizure-induced cell death throughout the lifespan of B6 mice (McCord et al., 2008).

MK-801 attenuates seizure-induced neuronal injury following kainate-induced status epilepticus

We examined differences in status-induced neuronal injury in young adult mice treated with kainate alone with those that received a single injection of the NMDA receptor blocker, MK-801, 90 minutes following kainate-induced SE. We found significant differences in the extent of injury between those mice that received the antagonist and those that received vehicle when cell death was measured 7 days later (Table 1; Fig. 1, Fig. 4). Irrespective of the hippocampal area examined, protection was only observed when the lower dose of MK-801 (0.5 mg/kg) was administered to young adult FVB mice. Post hoc analyses revealed a dramatic protective effect of MK-801 when administered after Kainate-induced SE in the dentate hilus

($P=0.003$), area CA3 ($P<0.001$), and in area CA1 ($P<0.001$). Treatment with MK-801 after kainate-induced SE also significantly reduced the severity of neuronal damage in several regions, including the amygdala, pyriform cortex, and thalamus (data not shown). In contrast, administration of the higher dose of MK-801 (1.0 mg/kg) 90 minutes following Kainate-induced SE was without effect on modulating susceptibility or resistance to KA in young adult FVB mice (Table 1; Fig. 1, Fig. 4).

In contrast to our observations in young adult mice, the lower dose of MK-801 (0.5 mg/kg) failed to protect hippocampal neurons of mature mice against seizure-induced cell death, irrespective of the hippocampal subfield examined. However, when MK-801 was administered at 1 mg/kg, 90 minutes following Kainate-induced status epilepticus, a significant protective effect was noted in all hippocampal subfields (Table 1; Fig. 2, Fig. 4). Similar to our observations in young adult mice, the most significant protective effect in mature mice was found within area CA3 ($P=0.001$).

Interestingly, the protective effects of MK-801 following Kainate-induced SE did not vary in a dose-dependent manner in aged mice (Table 1; Fig. 3, Fig. 4), as compared to the dose-dependent effect observed in mature mice. In particular, the lower dose of MK-801 (0.5 mg/kg) nearly completely protected dentate hilar neurons ($P<0.001$), and, as well, significantly reduced the extent of seizure-induced cell death in CA3 and CA1 pyramidal neurons. In contrast, the higher dose of MK-801 (1.0 mg/kg) seemed equally efficacious at reducing the extent of cell death, irrespective of the hippocampal subfield examined (Table 1; Fig. 3, Fig. 4). No statistically significant differences in the effectiveness of either dose at protecting any of the hippocampal subfields were noted. Both doses seemed equally efficacious at decreasing seizure-induced cell death.

Administration of NBQX suppressed the neural damage produced by KA

In contrast to our results with MK-801, we found significant differences in the ability of NBQX to block hippocampal cell death in the kainate model of temporal lobe epilepsy. In particular, while the lower dose of NBQX (30 mg/kg) was ineffective at modulating susceptibility or resistance to Kainate-induced cell death in young adult FVB mice (Table 2, Fig. 5, Fig. 8), post hoc analyses revealed a dramatic protective effect of the higher dose of NBQX (60 mg/kg) following Kainate-induced SE in both the dentate hilus ($P=0.001$), area CA3 ($P<0.001$), and a modest protective effect within area CA1 of young adult FVB mice ($P<0.001$).

The ability of NBQX to protect against seizure-induced cell death in mature mice was confounding. We found dose-dependent differences in the effectiveness of protection that were hippocampal subfield-dependent (Table 2, Fig. 6, Fig. 8). In particular, 30 mg/kg of NBQX was only protective in area CA1 (30% cell loss with NBQX as compared to 70% with vehicle + KA). However, the 60 mg/kg dose of NBQX was only effective at protecting dentate hilar neurons (30% cell loss with NBQX as compared to 80% with vehicle + KA). It is important to note that no statistically significant difference in the effectiveness was noted between the two doses of drugs within the dentate hilus, albeit a statistically significant protective effect was observed at the higher dose of NBQX (60 mg/kg) as compared to vehicle + KA-treated mice (Fig. 6). Surprisingly, NBQX was unable to protect the CA3 hippocampal subfield against Kainate-induced cell death, irrespective of the dose administered, in mature mice (Table 2; Fig. 6, Fig. 8).

In contrast, aged mice administered the high dose of NBQX (60 mg/kg) showed dramatic resistance to seizure-induced cell death following Kainate-induced SE (Table 2; Fig. 7, Fig. 8) in all hippocampal subfields examined. In particular, NBQX seemed equally efficacious at preventing significant cell damage within the dentate hilus ($P<0.001$), area CA3 ($P<0.001$), and area CA1 ($P<0.001$). It should be noted that we did not administer the lower dose of NBQX

(30 mg/kg) to aged mice based on results from an initial pilot study in which it appeared that the lower dose of NBQX was ineffective at modulating susceptibility or resistance to Kainate-induced cell death in both young adult mice and mature mice. Therefore, our aged animals only received the higher dose of NBQX.

Comparative neuroprotective effects of MK-801 and NBQX against kainate-induced SE

Comparison of the blockade of seizure-induced cell death by AMPA versus NMDA receptor antagonists, demonstrated differential effectiveness based on the age of the animal and hippocampal subfield examined. In particular, in young adult mice, either MK-801 or NBQX were equally efficacious at reducing seizure-induced cell damage following Kainate-induced SE (~1.8 fold reduction for 0.5 mg/kg MK-801 versus ~2.2 fold reduction for 60 mg/kg NBQX). A similar finding was reported for area CA3, with MK-801 at the low dose providing a nearly 14 fold reduction in the extent of cell death, while NBQX at the higher dose provided a nearly 11 fold reduction in cell death. For area CA1, MK-801 was more effective at reducing brain damage in that the low dose of MK-801 resulted in a nearly 10 fold reduction while NBQX at the highest dose modestly reduced cell death by ~ 2 fold.

Among mature mice, only within the dentate hilus, was either antagonist equally efficacious at reducing the extent of seizure-induced cell death. Both antagonists reduced cell death in a dose-dependent manner within the hilus, with the higher dose of either drug reducing cell death by nearly 3 fold. For both area CA3 and area CA1, MK-801 was more protective overall. Among mature mice, the higher dose of MK-801 was essentially able to completely protect neurons in areas CA3 and CA1 against seizure-induced cell death. In contrast, NBQX at either dose was unable to significantly protect against CA3 cell death following Kainate-induced status epilepticus, and the 30 mg/kg dose of NBQX was able to reduce cell death in area CA1 modestly.

In contrast, aged mice showed greater protection against seizure-induced cell death following administration of NBQX. While MK-801 at either dose was also protective against cell death irrespective of the hippocampal subfield examined, NBQX showed a much greater extent of protection in all hippocampal subfields (9.5 fold in the dentate hilus versus 7 fold for MK-801). The protective effects of NBQX were most dramatic within area CA3, in that 60 mg/kg NBQX reduced seizure-induced cell death by nearly 10 fold, while MK-801 at the most efficacious dose (1 mg/kg) only reduced cell death by 3.5 fold.

Discussion

We have previously shown that FVB mice susceptible to kainate-induced toxicity at a young age, exhibit greater excitotoxic damage with age (McCord et al., 2008), suggesting that susceptibility to the neurotoxic effects of kainate is regulated in a strain-and age-dependent manner. While the mechanisms underlying the resultant increased injury in aging brain remain unclear, we wanted to determine if alterations in the affinity or responsiveness of AMPA/KA or NMDA receptors to kainate administration might underlie or contribute to the enhanced susceptibility to age-related neuropathology. As an initial means to address whether receptor mechanisms are differentially affected in an age-dependent manner in this process, we examined if two glutamate receptor antagonists could attenuate seizure-induced cell death in the hippocampus following kainate-induced status epilepticus in young, mature and aged C57BL/6J (B6) and FVB/NJ (FVB) mice. In this study, we used antagonists of both NMDA and AMPA/KA subclasses of glutamate receptors (i.e. MK-801 and NBQX, respectively). MK-801 is a noncompetitive NMDA receptor antagonist that exhibits use dependency, blocking the ion channel only when an agonist is present and the ion channel is opened (Luer et al., 1996). NBQX is an analog of the quinoxaline dione antagonists, and is a potent and selective inhibitor of binding to the quisqualate subtype of glutamate receptors with no activity

at the NMDA or glycine sites (Sheardown et al., 1990). The present study found that receptor antagonists for both AMPA and NMDA reduced seizure-induced cell death in young, mature and aged FVB mice. Moreover, our results indicated that MK-801 had greater inhibitory effects on cell death in mature FVB mice, while NBQX was equally efficacious in young and aged FVB mice. In particular, NBQX resulted in a more prominent protective effect in aged FVB mice than that of MK-801.

In the present study, we found that Kainate-induced cell death was significantly attenuated by MK-801 in all age groups of FVB mice. In particular, a single dose of the NMDA receptor blocker, MK-801, significantly reduced the damage in limbic regions when given 90 minutes after kainate-induced SE. These results are in agreement with previous studies in which treatments with MK-801 were reported to reduce the neuronal damage after systemic injection of kainate or pilocarpine in rats (Fariello et al., 1989; Berg et al., 1993; Sutula et al., 1996; Yoshida et al., 1997; Rice and DeLorenzo, 1998; Dalby and Mody, 2001; Brandt et al., 2003). We chose to administer the NMDA receptor antagonist, MK-801, 90 minutes following kainate-induced status epilepticus, to determine if this regimen would be clinically practical. As well, this experimental design allowed us to test the ability of the antagonists to protect against the neuropathology induced by KA, while allowing us to nominally control for the amount of time spent in SE. While others have found that NMDA receptor antagonists given before or after the induction of KA-mediated seizures prevent most of the excitotoxic damage seen in limbic structures (Sperk, 1994; Brandt et al., 2003), these studies are based on the administration of a single dose of MK-801. Our studies found a dose-dependent effect between young and mature FVB mice, in that the lowest dose of MK-801 was only efficacious at reducing seizure-induced cell death in young adult FVB mice. In contrast, while the higher dose of MK-801 was without effect on reducing seizure-induced cell death in young adult FVB mice, it significantly reduced seizure-induced cell death throughout all hippocampal subfields in mature FVB mice. The significant suppression of hippocampal cell death observed when MK-801 was injected after KA strongly suggests that NMDA receptors have a crucial role in the determination of death for hippocampal cells, irrespective of age.

The present study found that treatment with the non-NMDA competitive antagonist, NBQX, showed suppression of seizure-induced cell death primarily in young adult and aged FVB mice, but only when the highest dose of NBQX was administered (60 mg/kg). The observation that NBQX post-treatment is effective against cell death induced by seizures suggests that AMPA receptor activation and subsequent Ca^{2+} influx may play a pivotal role in susceptibility to neuronal injury. AMPA receptor antagonists may indirectly inhibit Ca^{2+} entry through the NMDA receptor, the voltage-operated Ca^{2+} channel, and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Blockade of the AMPA receptor suppresses Na^+ - and K^+ -mediated cell depolarization and therefore prevents the opening of voltage-operated Ca^{2+} channels (Murphy and Miller, 1989). AMPA antagonists may also block Ca^{2+} entry through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, working in the reversed mode when the cell is depolarized. There is also evidence indicating that AMPA/KA receptors influence NMDA receptor-mediated responses by modulating the cell membrane potential (Young and Fagg, 1990; Raiteri et al., 1992). Thus, the neuroprotective effect of the AMPA receptor antagonist, NBQX, may be due at least in part to reduced activation of the NMDA receptor.

Surprisingly, we found that NBQX at a particular dose is not equally efficacious at protecting all hippocampal subfields against seizure-induced cell death in mature FVB mice induced by KA. In particular, while Kainate-induced hilar and CA1 pyramidal cell death was blocked by NBQX, albeit at two different doses of NBQX, NBQX, irrespective of dose, was unable to protect CA3 pyramidal neurons against seizure-induced cell death in mature FVB mice. The inability of the AMPA/KA receptor antagonist, NBQX, to attenuate seizure induced cell death in CA3 pyramidal neurons of mature FVB mice is neither a result of inadequate absorption of

the drug nor the testing of inappropriate doses. In the present study, NBQX was administered under conditions that were therapeutic in other animal models of kainate-induced seizures (Sheardown et al., 1990; Smith et al., 1991; Turski et al., 1992; Lallement et al., 1993) and in other ages. Although the number of AMPA receptors in the cortex and hippocampus are reduced depending on age (Le and Lipton, 2001; Nicolle et al., 1996; Magnusson and Cotman, 1993; Clark et al., 1992), the decreased effect of NBQX against CA3 hippocampal damage in mature FVB mice could be partially attributed to a more sensitive response via AMPA receptors. Moreover, the mechanism of this phenomenon may depend on receptor type. Therefore, when taken together, the data suggest that AMPA/KA receptors are not the primary mediators of seizure-induced cell death in the mature FVB mouse, at least within area CA3, although they may play an important role in other age groups. At present, the underlying reason for the inability of the AMPA/KA receptor antagonists to attenuate seizure-induced cell death of CA3 pyramidal neurons in mature FVB mice is difficult to explain.

One might expect differences between AMPA/KA vs. NMDA receptors to explain differences in the effectiveness of the antagonists in attenuating seizure-induced cell death in FVB mice. However, only in one age group, were we able to identify an area of divergence between the two receptor subtypes. In comparing the two antagonists, in mature FVB animals, MK-801 was more efficacious at protecting area CA3 and area CA1 against seizure-induced cell death, while both were equally efficacious at protecting dentate hilar neurons. We considered the possibility that convulsive doses of kainate preferentially interact with areas of the brain enriched with NMDA receptors, as compared to AMPA/KA receptors. However, the overlapping distribution of NMDA and AMPA/KA receptors in regions of the brain that underlie seizure-induced cell death, suggests that this is unlikely (Yin et al., 1999; Pickard et al., 2000; Watanabe et al., 2001). We found in the present study that Kainate-induced CA3 pyramidal cell death was blocked by MK-801 and NBQX both in young adult and aged FVB mice, suggesting that both NMDA and non-NMDA receptors are involved in Kainate-induced CA3 pyramidal cell death in at least these two age groups. These data support the hypothesis that responsiveness of the AMPA/KA receptor is altered during aging. At present, while no studies to date have examined hippocampal NMDA, AMPA or KA binding amongst inbred mouse strains throughout their life span, it is important to recognize that strain-dependent differences in the expression of glutamate receptor subunits may be involved in the selective vulnerability of certain strains to age-related excitotoxicity.

In conclusion, we found that both MK-801 and NBQX treatment reduced the neuronal damage produced by systemic kainate administration. However, NBQX was unable to protect against seizure-induced cell death in area CA3, irrespective of the dose, in mature (12 month old) FVB mice. Our results suggest that aging may differentially affect the ability of neuroprotectants to protect against hippocampal damage. Such age-related decreases in tolerance to antagonist administration may depend on a variety of factors including the expression and distribution of glutamate receptor subtypes. Moreover, differences in the effectiveness of these two antagonists in the aged animal could result from differential regulation of glutamatergic neurotransmitter systems or ion channel specificity with age. In particular, age-related differences may be influenced not only by differences in receptor density, but also by differences in receptor function and/or receptor kinetics.

In summary, the suppression of hippocampal cell death observed following treatment with either MK-801 or NBQX after kainate-induced SE strongly suggests that NMDA and AMPA/KA receptors are involved in the age-related regulation of seizure-induced excitotoxic neuronal death. As well, calcium homeostasis is altered during aging and changes in calcium channels have been reported to arise following status epilepticus, depending on the age of the animal examined (reviewed in Mattson, 2003; Raza et al., 2007). Further work is necessary to determine whether pharmacodynamic issues related to aging may play a significant role in

modifying the effectiveness of glutamate receptor antagonists in some of the hippocampal subfields across the age groups. However, in the present study, the highly selective AMPA/kainate receptor antagonist NBQX (Sheardown et al., 1990) and MK-801 were able to significantly attenuate the neurotoxic effects of kainate when administered alone, in FVB mice, in an age-specific manner.

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Young adult

Fig. 1.

Dose response effects of MK-801 on kainate-induced neuronal damage in hippocampal subfields of young adult FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of two doses of MK-801 (0.5, 1.0 mg/kg, i.p.) 90 minutes after Kainate-induced SE to young adult mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration and were dependent on the dose of antagonist. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Mature

Fig. 2.

Dose response effects of MK-801 on kainate-induced neuronal damage in hippocampal subfields of mature FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of two doses of MK-801 (0.5, 1.0 mg/kg, i.p.) 90 minutes after Kainate-induced SE to mature mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration and were dependent on the dose of antagonist and hippocampal subfield. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Fig. 3.

Dose response effects of MK-801 on kainate-induced neuronal damage in hippocampal subfields of aged FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of two doses of MK-801 (0.5, 1.0 mg/kg, i.p.) 90 minutes after Kainate-induced SE to aged mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration and were dependent on the dose of antagonist. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Fig. 4. MK-801 protects against limbic damage caused by kainate-induced status epilepticus in three age groups of mice. Corresponding low-power photomicrographs of NeuN-stained horizontal sections of the hippocampus showing differential cell loss 7 days after kainate-induced SE (vehicle), and in a representative mouse treated with 0.5 mg/kg MK-801, and a representative mouse treated with 1.0 mg/kg MK-801. Note the massive loss of neurons, as evidenced by loss of immunostaining, in the CA3 and CA1 fields of the hippocampus after S.E., which was prevented by MK-801. Hippocampal cell death was substantially reduced by MK-801 administration in all age groups in a dose-dependent manner. CA1 and CA3 denote the hippocampal subfields; H, dentate hilus. Scale bar = 750 μ m.

Young adult

Fig. 5.

Dose-response effects of NBQX on kainate-induced neuronal damage in hippocampal subfields of young adult FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of two doses of NBQX (30, 60 mg/kg, i.p.) 90 minutes after Kainate-induced SE to young adult mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration and were dependent on the dose of antagonist. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Fig. 6. Dose-response effects of NBQX on kainate-induced neuronal damage in hippocampal subfields of mature FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of two doses of NBQX (30, 60 mg/kg, i.p.) 90 minutes after Kainate-induced SE to mature mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration and were dependent on the dose of antagonist and hippocampal subfield. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Fig. 7. Effect of NBQX on kainate-induced neuronal damage in hippocampal subfields of aged FVB (susceptible) mice. Quantitative analysis of neuronal density in three hippocampal subfields following administration of NBQX (60 mg/kg, i.p.) 90 minutes after Kainate-induced SE to aged mice. Viable surviving neurons were estimated by cresyl violet staining. Bars denote the percentage of surviving neurons (as compared with saline-injected control mice) in each hippocampal region. Differences in the extent of cell loss were observed 7 days following kainate administration. Data represent the mean $\pm$ S.E.M. of at least 6 mice/treatment. Asterisks denote significant differences to B6 mice administered kainate ($P < 0.05$).

Fig. 8.

NBQX protects against limbic damage caused by kainate-induced status epilepticus in three age groups of mice. Corresponding low-power photomicrographs of NeuN-stained horizontal sections of the hippocampus showing the differential cell loss 7 days after kainate-induced SE (vehicle), and in a representative mouse treated with 30 mg/kg NBQX, and a representative mouse treated with 60 mg/kg NBQX. Note the extensive loss of neurons in the dentate hilus, CA3 and CA1 subfields of the hippocampus after status epilepticus (vehicle), which was substantially reduced following administration of the highest dose of NBQX in young and aged mice. CA1 and CA3 denote the hippocampal subfields; H, dentate hilus. Scale bar = 750 μ m.

Table 1

Age-response comparison of MK-801 on hippocampal cell loss (percentage) following kainate administration in FVB mice

Hippocampal subregion Aged	Dose (mg/kg) P-value	Young Adult	Mature
Hilus	0	61.3±5.7	76.1±16.0
94.6±5.4	0.067		0.5 14.9±
2.4	55.7±4.6	13.8±5.7*	0.006
	1.0	59.0±11.3	28.1±9.9
33.6±7.6	0.103		
CA3	0	60.5±4.8	79.3±11.7
80.8±7.5	0.108		
	0.5	4.2±0.9	62.6±16.8
42.9±1.1*	0.004		
	1.0	78.5±6.0	4.7±0.4
23.3±2.4*	<0.001		
CA1	0	45.9±1.9	65.5±16.2
72.4±6.7	0.113		
	0.5	4.5±0.3	77.8±19.6
42.2±2.2*	0.005		
	1.0	72.5±12.3	9.0±5.5
20.1±1.9*	<0.001		

Values represent means ± S.E.M. of at least 5 mice in each age group.

* P<0.05 as compared to other age groups.

Table 2

Age-response comparison of NBQX on hippocampal cell loss (percentage) following kainite administration in FVB mice

Hippocampal subregion Aged	Dose (mg/kg) P-value	Young Adult	Mature
Hilus	0	66.1±5.7	76.1±16.0
94.6±5.4	0.067	30	50.5±
1.8	59.4±1.5*	ND	0.006
	60	16.5±4.1	26.7±3.1
10.0±4.5*	0.030		
CA3	0	60.5±4.8	79.3±11.7
80.8±7.5	0.108		
	30	65.2±1.1	65.1±10.4
ND	0.188		
	60	0.50±1.0	71.5±12.1
7.9±3.9*	<0.001		
CA1	0	45.9±1.9	65.5±16.2
72.4±6.7	0.113		
	30	62.9±4.6	33.1±2.4*
ND	<0.001		
	60	25.0±4.5	66.9±7.1
6.2±2.2*	<0.001		

Values represent means ± S.E.M. of at least 5 mice in each age group.

* P<0.05 as compared to other age groups.