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Research Paper

NBQX, a highly selective competitive antagonist of AMPA and KA ionotropic glutamate receptors, increases seizures and mortality following picornavirus infection

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ABSTRACT

Seizures occur due to an imbalance between excitation and inhibition, with the balance tipping towards excitation, and glutamate is the predominant excitatory neurotransmitter in the central nervous system of mammals. Since upregulation of expression and/or function of glutamate receptors can contribute to seizures we determined the effects of three antagonists, NBQX, GYKI-52466 and MK 801, of the various ionotropic glutamate receptors, AMPA, NMDA and KA, on acute seizure development in the Theiler's murine encephalomyelitis virus (TMEV)-induced seizure model. We found that only NBQX had an effect on acute seizure development, resulting in a significantly higher number of mice experiencing seizures, an increase in the number of seizures per mouse, a greater cumulative seizure score per mouse and a significantly higher mortality rate among the mice. Although NBQX has previously been shown to be a potent anticonvulsant in animal seizure models, seizures induced by electrical stimulation, drug administration or as a result of genetic predisposition may differ greatly in terms of mechanism of seizure development from our virus-induced seizure model, which could explain the opposite, proconvulsant effect of NBQX observed in the TMEV-induced seizure model.

1. Introduction

Seizures occur due to an imbalance between excitation and inhibition with the balance tipping towards excitation (Nadler, 2012). Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS) of mammals with fully 60–70% of all synapses being glutamate synapses (Nadler, 2012). Therefore upregulation of expression and/or function of glutamate receptors can contribute to seizures (Dingledine, 2012; Nadler, 2012). There are three subfamilies of ionotropic glutamate receptors, named based on their activation by selective agonists: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate), NMDA (*N*-methyl-D-aspartate) and KA (kainate) receptors (Dingledine, 2012). AMPA, NMDA and KA receptors are expressed on microglia, astrocytes, oligodendrocytes, and neurons (Gottlieb and Matute, 1997; Hagino et al., 2004; Lees, 2000; Matute, 2006; Murugan et al., 2011; Nadler, 2012; Noda et al., 2000). The region of the brain with the highest density of NMDA and AMPA receptors is the hippocampus, specifically CA1 for AMPA receptors (Rainbow et al., 1984; Wong et al., 1986). KA receptors are found to be most abundant in brain regions where NMDA receptors are least abundant (Nadler, 2012). Nevertheless, hippocampal neurons have been shown to express KA receptors (Paternain et al., 1995). The kinetic properties of KA receptors are intermediate between AMPA and NMDA receptors and there is considerable overlap in agonist action between KA and AMPA receptors (Nadler, 2012). Antagonists to the various ionotropic glutamate receptors, used in this study, include: NBQX [2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline], a highly selective competitive antagonist of AMPA and KA receptors (Sheardown et al., 1990); GYKI-52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine], a non-competitive antagonist, or negative allosteric modulator, of AMPA receptors (Lees, 2000; Wilding and Huettner, 1995); and MK 801 [(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine], also known as Dizocilpine, a potent non-competitive antagonist of NMDA receptors (Rod and Auer, 1989;

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; ANOVA, Analysis of variance; ASDs, anti-seizure drugs; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CNS, central nervous system; DA, Daniels; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GYKI-52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; GYKI-53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5*H*-2,3-benzodiazepine; i.c., intracerebrally; i.p., intraperitoneal; KA, kainate; MK 801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; mTLE, mesial temporal lobe epilepsy; NBQX, 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; pfu, plaque-forming units; p.i., post-infection, PLSD, Protected Least Significant Difference; RCA, *Ricinus communis* agglutinin, SE, status epilepticus; SEM, standard error of the mean, TMEV, Theiler's murine encephalomyelitis virus.

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Wong et al., 1986). NBQX, GYKI-52466 and MK 801 all readily penetrate into the CNS when administered peripherally (Sheardown et al., 1990; Smith et al., 1991; Vizi et al., 1996; Wong et al., 1986).

Previous studies have shown that NBQX, GYKI-52466 and MK 801 block seizures in various animal seizure models (Barton et al., 2003; Chapman et al., 1991; Loscher and Honack, 1994; Twele et al., 2015). We determined the effects of these antagonists on acute seizure development in the Theiler's murine encephalomyelitis virus (TMEV)-induced seizure model, an infection-driven animal model for epilepsy [reviewed in (Libbey and Fujinami, 2011)]. In this model, approximately 50% of C57BL/6J mice infected with TMEV experience acute behavioral seizures between days 3 and 10 post-infection (p.i.) (Libbey et al., 2008; Stewart et al., 2010b). The infected mice clear the virus by about day 14 p.i. (Kirkman et al., 2010; Libbey et al., 2011b, 2010), and then approximately 50% of the mice that had acute seizures develop spontaneous seizures (epilepsy) following a latent period (Stewart et al., 2010a). Hippocampal neurons, particularly within the CA1 region, are infected and undergo cell death (Kirkman et al., 2010; Libbey et al., 2008; Stewart et al., 2010b).

We found that, of the three antagonists of ionotropic glutamate receptors tested, only NBQX had an effect on acute seizure development in the TMEV-induced seizure model. Treatment with NBQX resulted in a significantly higher number of mice experiencing seizures, an increase in the number of seizures per mouse, a greater cumulative seizure score per mouse and a significantly higher mortality rate among the mice. Therefore, although NBQX has previously been shown to be a potent anticonvulsant, it has the opposite effect in the TMEV-induced seizure model. It should be noted that animal seizure models in which the seizures are induced by electrical stimulation, drug administration or as a result of genetic predisposition may differ greatly in terms of mechanism of seizure development from our virus-induced seizure model.

2. Methods

2.1. Animal experiments

Four week old, male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were reviewed and approved by the University of Utah Institutional Animal Care and Use Committee (Protocol #12-09006) and conducted in accordance with the guidelines prepared by the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council. All animal studies complied with the ARRIVE guidelines. All efforts were made to minimize suffering. Mice were euthanized through an overdose of isoflurane.

2.2. TMEV infection

C57BL/6J mice were anesthetized with isoflurane by inhalation and infected intracerebrally (i.c.) with 3×10^5 plaque-forming units (pfu) of the Daniels (DA) strain of TMEV at a final volume of 20 μl per mouse. The site of injection was in the postparietal cortex of the right cerebral hemisphere to a depth of 2 mm [posterior (caudal) and medial of the right eye at approximately bregma −2 mm and interaural + 8 mm] (Kirkman et al., 2010). The needle had a William's collar to limit penetration of the tip to 2 mm. The DA strain of TMEV was propagated as previously described (Tsunoda et al., 1997).

2.3. Drug treatment

TMEV-infected mice were treated, via intraperitoneal (i.p.) injection, with MK 801 (1 mg/kg twice daily, Sigma, St. Louis, MO), GYKI-52466 (10 mg/kg twice daily, Sigma) (Nargi-Aizenman et al., 2004) or NBQX (approximately 22.5 mg/kg twice daily, Alomone Labs, Jerusalem, Israel) (Docagne et al., 2007), all in a 25 μl volume, starting on day 2.5 p.i. and stopping on day 10.5 p.i. These doses are tolerated and considered sufficient to block ionotropic glutamate receptor transmission in an efficient and specific manner. Control infected mice were i.p. injected with 25 μl phosphate-buffered saline (PBS).

2.4. Seizure scoring

C57BL/6J mice infected with DA were weighed and monitored daily for seizures through day 21 p.i. The monitoring of seizure activity was performed as previously described (Libbey et al., 2011a). Briefly, mice were observed for 2 h each day immediately following the first injection of antagonist of the day. However, if a mouse experienced a new seizure (i.e. the mouse had not previously been observed having a seizure) immediately following the second injection of antagonist of the day, then the seizure was scored and recorded. Mice were scored for 1 seizure per mouse per day. Seizure activity was graded using the Racine scale: stage 1, mouth and facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; stage 5, rearing and falling (Benkovic et al., 2004;Racine, 1972). Seizure burden was analyzed by assessing both seizure incidence (numbers of mice having seizures, numbers of observed seizures per mouse) and seizure severity [cumulative seizure score per mouse, numbers of mice with the various maximum seizure scores (stages 1–5), numbers of seizures scored as stage 5].

2.5. Histology

Mice were euthanized on day 21 p.i. and perfused with PBS, followed by 4% paraformaldehyde solution. Brains were harvested, divided into 5 coronal slabs, embedded in paraffin and cut into 4 μm thick tissue sections. Paraffin sections were stained with Luxol fast blue and analyzed for neuron loss in the hippocampus as previously described (Kirkman et al., 2010). Slides were examined in a blinded fashion using one slide, containing sections from all 5 coronal slabs, per brain ($N = 14$ to 20 brains per experimental group for antagonist-treated mice and 55 brains per experimental group for PBS-treated mice). The tissue section of only 1 of the 5 coronal slabs per slide contained the hippocampal region of the brain. The extent of neuron loss in the pyramidal cell layer of the hippocampus from CA1 to CA3 was given a graded score as follows: score 0, no damage; score 1, 10–29% cell loss; score 2, 30–59% cell loss; and score 3, >60% cell loss. A score was given for each of the two hippocampi present in a brain and then the scores were summed so the highest possible score for neuron cell loss per brain could be 6 (the highest score, 3, for two regions of the brain).

Viral antigen positive cells were detected on consecutive paraffin sections using TMEV hyperimmune rabbit serum, as previously described (Tsunoda et al., 2001; Zurbriggen and Fujinami, 1989). Astrocytes were detected on paraffin sections using glial fibrillary acidic protein (GFAP) antibody (DAKO Corp., Carpinteria, CA), as previously described (Kirkman et al., 2010). The slides were labeled using the avidin-biotin peroxidase complex technique with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.01% hydrogen peroxide (Sigma) in PBS. Enumeration of viral antigen positive cells was performed in a blinded fashion with a light microscope using one slide per brain and evaluating the tissue section containing the hippocampal region ($N =$ 15 to 20 brains per experimental group for antagonist-treated mice and 55 brains per experimental group for PBS-treated mice). Viral antigen positive cells were enumerated and summed from each of the two hippocampi present in a brain and each of the two dentate gyri present in a brain. The extent of gliosis was semi-quantified by scoring $GFAP^+$ activated astrocytes in the hippocampus and dentate gyrus in a blinded fashion using one slide per brain ($N = 14$ to 20 brains per experimental group for antagonist-treated mice and 54 brains per experimental group for PBS-treated mice). Activated astrocytes have larger cell bodies, fatter processes, and stain more intensely for GFAP than quiescent astrocytes. Gliosis was given a graded score as follows: score 0, ≤50 activated astrocytes present; score 0.5, 51–168 activated astrocytes; score 1, 169–285 activated astrocytes; score 1.5, 286–402 activated astrocytes; score 2, 403–519 activated astrocytes; score 2.5, 520–636