Anticonvulsant Action of GABA in the High Potassium – Low Magnesium Model of Ictogenesis in the Neonatal Rat Hippocampus

in vivo and in vitro

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ABSTRACT

Previous developmental studies in vitro suggested that the inhibitory neurotransmitter GABA exerts depolarizing and excitatory actions on the immature neurons and that depolarizing GABA is causally linked to ictal activity during the first weeks of postnatal life. However, remarkably little is known on the role of GABA in the generation of neonatal seizures in vivo. Here, using extracellular recordings from CA3 hippocampus, we studied the effects of GABA(A) acting drugs on electrographic seizures induced by local intrahippocampal injection of the epileptogenic agents (high-K⁺/low Mg²⁺) in the non-anaesthetized rats in vivo and in the hippocampal slices in vitro during the second postnatal week (postnatal days P8-12). We found that in vivo, the induction of ictal-like events was facilitated by co-infusion of high-K⁺/low Mg²⁺ together with the GABA(A) antagonist bicuculline or gabazine. Moreover, the infusion of bicuculline alone caused ictal-like activity in about 30% of cases. Co-infusion of the GABA(A) receptor agonist isoguvacine or the GABA(A) positive allosteric modulator diazepam completely prevented high-K⁺/low Mg²⁺- induced seizures. In in vitro studies using hippocampal slices we also found that high-K⁺/low Mg²⁺ produced ictal activity that was exacerbated by bicuculline and gabazine and reduced by isoguvacine. Thus, in the model of high-K⁺/low Mg²⁺ induced seizures both in in vivo and in vitro conditions, GABA, acting via GABA(A) receptors, has an anticonvulsant effect during the critical developmental period of enhanced excitability.

Keywords: Epilepsy, Seizure, Inhibition, Neonate, Rat
INTRODUCTION

The neonatal brain is quite prone to seizure (Holmes 1994; Holmes et al. 2002). These clinical findings are paralleled by laboratory observations in lower animals. A critical period of seizure susceptibility – the second postnatal week in the rat - has been documented with various epileptogenic agents and conditions including kainic acid (Tremblay et al. 1984; Albala et al. 1984), electrical stimulation (Moshe et al. 1981), hypoxia (Jensen et al. 1991), penicillin (Swann and Brady, 1984), picrotoxin (Gomez-Di Cesare et al. 1997), fever (Holtzman et al. 1981; Baram et al. 1997), GABA(B) receptor antagonists (McLean et al. 1996) and increased extracellular potassium (Dzhala and Staley 2003; Khazipov et al. 2004). Although several hypothesis have been put forward to explain the increased excitability during this period of life (for reviews, see Baram and Hatalski 1998; Holmes and Ben-Ari 1998; Swann and Hablitz 2000), the underlying mechanisms are not completely understood. Recently it has been demonstrated that the critical period of enhanced excitability lays within the time window when GABA exerts paradoxical excitatory action, raising a hypothesis that enhanced excitability is due to the inversed – excitatory instead of inhibitory – actions of GABA (Dzhala and Staley 2003; Khazipov et al. 2004).

GABA is the principal inhibitory neurotransmitter in the adult brain. However, at early developmental stages GABA acting via GABA(A) receptors may exert excitatory effects (Ben-Ari et al. 1989, 1997, 2002). The excitatory effects of GABA are due to elevated intracellular concentration of chloride ions because of delayed expression of the chloride extruder KCC2 resulting in a depolarized value of GABA(A)-mediated conductance (Rivera et al. 1999). In addition to the excitatory effects, depolarizing GABA may also exert inhibitory actions on immature neurons via a shunting mechanism (Chen et al. 1996; Khalilov et al. 1999; Lamsa et al. 2000; Wells et al. 2000; Lu and Trussell 2001). Thus, GABA in the immature brain plays both an excitatory and inhibitory roles. Hence the complexity in the GABAergic contributions to neuronal network phenomena including paroxysmal discharges. While mainly antiepileptic
effects of GABA have been documented thus far in the immature brain (Smythe et al. 1988; Kubova and Mares 1991; Velisek et al. 1995; Kubova et al. 1999), recent studies using a high-potassium model of ictogenesis in the hippocampal slices from neonatal rats suggested that excitatory GABA may be causally linked to ictal activity in the early developmental window (Dzhala and Staley 2003). The GABA(A) antagonists bicuculline and gabazine completely suppressed high-potassium induced ictal-like events, while GABA(A) agonists muscimol and isoguvacine significantly increased the frequency and duration of the ictal events (Dzhala and Staley 2003). In another study using a similar model (Khazipov et al. 2004), blockade of GABA(A) receptors resulted either in a blockade or reduction in frequency of high potassium induced ictal events, in conformation of the Dzhala and Staley (2003) result, and further suggesting a proconvulsant role for GABA; however, GABA(A) antagonist also caused a significant increase in the amplitude of population spikes, suggesting a coexisting anticonvulsant role for GABA during the critical development period.

While slices can provide valuable information, extrapolation of the results obtained in vitro to the in vivo situation may often be difficult in particular when it comes to complex network phenomena (Steriade 2001). Therefore, in the present study we studied the effects of the GABA(A) acting drugs on epileptiform activity induced by high-K+/low Mg²⁺ in the hippocampus of postnatal rats in vivo and hippocampal slices in vitro. We found that blockade of GABA(A) receptors significantly facilitates seizures induced by intrahippocampal injection of high-K+/low Mg²⁺ solution whereas the GABA(A) promoting drugs – the agonist isoguvacine and positive allosteric modulator diazepam – completely prevents the epileptiform activity. Similar proconvulsive actions of the GABA(A) antagonists and anticonvulsive actions of the GABA(A) promoting drugs were also found in the hippocampal slices in vitro. It appears that in the high-K⁺/low Mg²⁺ model of ictogenesis, GABA acting via GABA(A) receptors has an anticonvulsant role during the critical developmental period of enhanced excitability.
MATERIALS AND METHODS

Animal preparation

Sprague-Dawley rat pups (n=36) of postnatal days [P] 8-12 and 6 rat pups at P4-P6 were used for in vivo part of the study and were treated in accordance with the guidelines set by the National Institute of Health and Dartmouth Medical School for the humane treatment of animals as described in details previously (Khazipov et al., 2004). In brief, animals were anesthetized with isoflurane (induction 4%, maintenance 2%) in an O₂ carrier using an agent-specific vaporizer (Isotec 3, Ohmeda Medical System). For general analgesia Buprenex (0.01 to 0.02 mg/kg buprenorphine hydrochloride) was administered subcutaneously. Skin, subcutaneous fat, and periosteum were removed from the skull, which then was covered with a thin layer of dental acrylic except for an area of approximately 2 mm in diameter above the hippocampus and small area above the cerebellum for placement of recording and reference electrodes. Two anchor bars were attached to the frontal and the occipital bones of the skull with dental acrylic. Rats were placed in the cotton nest with the head restrained in the stereotaxic apparatus by the skull bars. The body temperature was maintained constant at 35°C using a heater (Warner Instrumental Corp., CT).

A burr hole of 0.5 mm in diameter was drilled in the skull above the hippocampus. The dura was cut and removed. A wire electrode (50 μm in diameter; California Fine Wire, Grover Beach, CA) for extracellular field potential recordings was inserted into the application cannula (0.2 mm diameter, Plastic One Inc., Roanoke, VA). The tip of the recording electrode was extended for about 100 μm from the cannula ending. The application cannula with recording electrode was positioned into the CA3 pyramidal cell layer of the hippocampus under stereotaxic and electrophysiological guidance (2.0–2.5 mm caudal to bregma; 2.0–2.5 mm from midline; depth 2700-3100 μm). Reference and ground electrodes were implanted into the cerebellum. After surgery the isoflurane anesthesia was stopped and the pups were left to recover from anesthesia for 10-15 minutes, and then electrophysiological data were recorded uninterrupted for 60-120 min.
Electrical signals were amplified (x1000) with filter settings of 0.1–5000 Hz using a differential amplifier (A-M Systems, Carlsborg, WA) and digitized at 10 kHz using an analogue-to-digital converter (Digidata 1322A; Axon Instruments). Off-line analysis of the hippocampal electrogram was performed using Clampfit (Axon Instruments) and Origin 5.0 (Micrcal Software, Northampton, MA). Group data are expressed as means ± SEM; error bars also indicate SEM.

Interictal-like activity was defined as brief (80-200ms) high amplitude spikes in the EEG that occurred in isolation on a background of otherwise normal activity. Ictal-like activity consisted of rhythmic spikes. Tonic was arbitrary used to describe sustained rhythmic spikes whereas clonic was defined as bursts of rhythmic spikes interspersed with lower amplitude, non-rhythmic activity (McCormick and Contreras 2001). The tonic and clonic rhythms refer to the EEG correlates to the tonic and clonic behaviors observed during seizures. In this study we used these terms arbitrarily to describe the EEG patterns that typically occur in conjunction with behavioral seizures. The term epileptiform discharges was used to describe either ictal or interictal activity.

After the recordings, the rat was anesthetized, and the brain was removed. Sagital 300 µm slices were cut using a vibroslicer Leica VT 1000S (Leica Microsystems, Nussloch GmbH, Germany). Electrode position verification was performed under light microscopic evaluation.

**Slice preparation**

Sprague-Dawley rat pups (n=6) of postnatal days [P] 8-12 were prepared as described (Khazipov et al., 2004). In brief, rats were deeply anaesthetized using isoflurane and decapitated. The brain was removed and placed into ice-cold ‘solution’ of the following composition (mM): sucrose 250, KCl 2, CaCl₂ 0.5, MgCl₂ 7, NaHCO₃ 26, NaH₂PO₄ 1.2 and glucose 11 (pH=7.4). Transverse hippocampal slices were cut using the Leica 1000S vibroslicer (Leica Microsystems, Nussloch GmbH, Germany). After dissection, slices were kept in an oxygenated (95 % O₂-5% CO₂) artificial cerebrospinal fluid (ACSF) solution of the following composition (mM): NaCl 126, KCl 3.5, CaCl₂
2.0, MgCl₂ 1.3, NaHCO₃ 25, NaH₂PO₄ 1.2 and glucose 11 (pH 7.3) at 30-32 °C for at least 1.5 h before use.

**Electrophysiological recordings**

For recordings, slices were transferred to a submersion-type thermostatic chamber (Warner Instrument Corp., Hamden, CT, USA) and superfused at 30-32°C at a rate of 1-3mL/min with the oxygenated ACSF. Extracellular recordings were made from CA3 pyramidal cell layer using borosilicate glass pipettes. The patch electrodes were made from borosilicate glass capillaries (GC150F-15, Clark Electromedical Instruments) and filled with extracellular solution. Pipette resistances ranged from 5-7 MΩ. The recordings were performed using an Axopatch 200A amplifier (Axon Instruments) and digitized (10 kHz) online with an analogue-to-digital converter Digidata 1322A (Axon Instruments).

**Administration of drugs**

For the *in vivo* experiments drugs were applied locally through the stainless-steel cannula (inner diameter 100 µm) using microsyringes. Drugs were dissolved in the ACSF. In the experiments with high-potassium and low magnesium, 10 mM KCl was substituted for equivalent concentrations of NaCl. MgCl₂ was omitted from the ACSF. Application was made by repetitive injection of 5 µl-volumes (duration: 10-15 s; interval: 5 minutes; number: up to 10 times). Repetitive microinjections were chosen instead of single continuous injection to avoid displacement of the recording electrode.

In the *in vitro* experiments, high-K⁺/ low Mg²⁺ was applied directly to the perfusion solution. When assessing the role of GABA(A)-acting drugs on seizure induction the slices were incubated with isoguvacine, bicuculline, gabazine or diazepam for 10 minutes prior to application of high-K⁺/ low Mg²⁺. We varied the sequence of drugs application to avoid use-dependent effects.

**Chemicals**

6-Cyano-7-nitroquinoxaline-2,3-dione(CNQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-APV), isoguvacine, bicuculline and SR 95531 hydrobromide (gabazine) were obtained
from Tocris (Ellisville, MO, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

**RESULTS**

We recorded local field potentials from the CA3 pyramidal cell layer of the hippocampus in P8-P12 Sprague Dawley rats. Hippocampal seizures were induced by brief repetitive microinjections (5 µl administered ten times at 5 minute intervals) of the high-K⁺ (10 mM) / low Mg²⁺ ACSF into the stratum radiatum of CA3 hippocampus in the vicinity of the recording electrode (separation distance ≈ 100 µm) (Fig. 1A). Seizures occurred after the 5-9th microinjection (6.8±1.6; n= 5 rats; Fig. 1D). Electrographic seizures had typical ictal morphology with an initial phase of initial bursting discharge followed by the tonic- (4-10 Hz) and clonic-like discharges (Fig. 1B and C). Once induced, seizures were generated in response to each additional microinjection. The duration of the interictal and ictal discharges did not change significantly with the serial injections of high-K⁺/low Mg²⁺ solution. Injection of the high-K⁺ (10 mM) / low Mg²⁺ ACSF in young rat pups (P4-P6) (n = 6) did not lead to electrographic seizures (not shown).

We further studied the pharmacology of seizures by co-injection of various drugs together with high-K⁺/low Mg²⁺ solution. Addition of the AMPA/kainate receptor antagonist CNQX (10 µM; n=5 rats) or NMDA receptor antagonist D-APV (50 µM; n=5 rats) completely prevented seizure occurrence (Fig. 3) in keeping with the pivotal role of glutamatergic neurotransmission in ictogenesis.

Using similar experimental approaches we further studied GABAergic modulation of seizures by co-injection of the GABA(A) acting drugs in conjunction with epileptogenic solution. Co-application of the high-K⁺/low Mg²⁺ solution together with the GABA(A) receptor antagonist bicuculline (15 µM; n=5) or gabazine (10µM; n=4) reliably caused seizures in all animals (Fig. 2). Moreover, seizures began significantly earlier compared to the application of high-K⁺/low Mg²⁺ solution without bicuculline/gabazine, occurring within 1-3 microinjections (2.0±0.7; n=5;
The injection of 15 µM bicuculline alone caused seizures in about a 30% of animals (2 of 6 rats).

The GABA(A) receptor agonist isoguvacine (10 µM), co-applied together with high-K⁺/low Mg²⁺ solution completely prevented seizures occurrence (Fig. 3; n= 5 rats). Seizures were also completely prevented by co-application of the positive allosteric modulator of the GABA(A) receptors diazepam (5 µM) with the high-K⁺/low Mg²⁺ solution (Fig. 3; n= 5 rats). Systemic injection of diazepam (5 mg/kg) also completely prevented the appearance of seizures in high-K⁺/low Mg²⁺ ACSF solution (n=3).

In the next series of experiments we studied the effects of GABA(A) acting drugs on the high-K⁺/low Mg²⁺ induced epileptiform activity in the P8-12 hippocampal slices in vitro (Fig. 4). Using extracellular field potential recordings form CA3 pyramidal cell layer we found that brief application of the high-K⁺/low Mg²⁺ ACSF readily induces ictal-like tonic-clonic discharges in all slices studied (n=11)(Fig. 4A). Maximal amplitude and frequency of the population spikes were 1.2±0.2 mV and 9.2±1.1 Hz during the epileptiform discharges. In the presence of 10 µM isoguvacine, application of high-K⁺/low Mg²⁺ solution also caused epileptiform activity in P8 slices but both the maximal amplitude and frequency of population spikes during the discharges were significantly reduced by 25±3% and 42±3%, respectively (n=6) (Fig. 4C). In P12 slices, isoguvacine (10 µM) completely prevented the generation of epileptiform discharges in response to high-K⁺/low Mg²⁺ solution (n=4). Bicuculline (15µM) (n=6) and gabazine (n=5) (10µM) increased the maximal amplitude of the population spikes by 26±3% as well as the maximal frequency of the population spikes by 53±4% (for bicuculline, n=3 at P8 and n=3 at P12; for gabazine, n=3 at P8 and n=2 at P12 slices) (Fig. 4B). Application of diazepam 10µM did not significantly change the amplitude or frequency of discharges evoked by application of 10mM K⁺/low Mg²⁺ solution (n=4; n=2 at P8 and n=2 at P12).

**DISCUSSION**
In the present study we attempted to determine the role of GABA(A) receptors in the generation of hippocampal seizures during the critical developmental period of enhanced excitability in the high K⁺/low Mg²⁺ model of ictogenesis in vivo and in vitro.

We found that blockade of GABA(A) receptors with bicuculline and gabazine facilitated high-K⁺/low Mg²⁺ induced seizures and bicuculline also induced seizures in about 30% of cases in vivo. We also found that isoguvacine, which activates GABA(A) receptors directly, as well as diazepam, which enhances GABA(A) mediated responses evoked by endogenous GABA, efficiently inhibit seizures induced by high-K⁺/low Mg²⁺ solution in vivo. Similar proconvulsive effects of the GABA(A) blockers and anticonvulsive effects of the GABA(A) agonist were observed in the model of high-K⁺/low Mg²⁺ - induced ictogenesis in the hippocampal slices at P8-12 rats in vitro. The present results are consistent with an anticonvulsant role of GABA during early brain development.

The role of GABA in ictogenesis during the first weeks of life has been previously studied in different in vitro and in vivo models but the conclusions differ. It has been demonstrated that blockade of GABA(A) receptors induces interictal- and ictal-like activities (Khalilov et al. 1997, 1999; Lamsa et al. 2000; Wells et al. 2000; Swann and Brady 1984; Gomez-Di Cesare et al. 1997; Quilichini et al. 2002, 2003) and aggravates seizure-like activity induced by other epileptogenic agents and conditions in various hippocampal and neocortical preparations from birth onwards. GABA(A) agonists and positive modulators also typically suppress epileptiform activity in the immature cortex (Quilichini et al. 2002, 2003). Yet in several models of ictogenesis, including the acute high-potassium model in hippocampal slices (Dzhala and Staley 2003; Khazipov et al. 2004a) and mirror focus in the intact hippocampus in vitro model (Khalilov et al. 2003), blockade of GABA(A) receptors reduces the frequency or even completely suppresses ictal-like events whereas GABA(A) agonists increases the frequency and duration of the ictal-like events (Dzhala and Staley 2003). Interestingly, the “proepileptic” actions of GABA have been also observed in several in vitro models of ictogenesis in adults. It has been shown that during seizure-like activity in slices from adult animals, a dynamic switch occurs in the action of GABA from inhibitory to excitatory as a result of massive release of
GABA (Avoli et al. 1996; Lopantsev and Avoli 1998) and that GABA(A) mediated excitation contributes substantially to neuronal synchronization during seizure-like events in the low-magnesium model (Kohling et al. 2000). Exposure of a rat hippocampal slice to GABA(B) receptor antagonists in the absence of ionotropic glutamatergic transmission leads to a progressive synchronization of spontaneous interneuronal activity (Uusisaari et al. 2002). Fujiwara-Tsukamato et al. (2003) have shown that GABAergic excitation participates in the expression of seizure-like rhythmic synchronization (afterdischarge) in the mature hippocampal CA1 region. Involvement of depolarizing GABA in the generation of interictal activity has been also shown in slices from patients with temporal lobe epilepsy (Cohen et al. 2002). On the other hand, in intact adult animals in vivo, GABA(A) receptor agonists and modulators, which enhance GABA(A) action typically exert anticonvulsive actions (Sankar and Holmes 2004).

Diversity in the roles for GABA in different models of ictogenesis may partly reflect dualism in the actions of depolarizing GABA. Even though GABA has a depolarizing and excitatory effect, GABA may also exert inhibitory function due to a shunting mechanism (Chen et al. 1996; Khalilov et al. 1999; Lamsa et al. 2000; Wells et al. 2000; Lu and Trussell 2001). The net effect of GABA depends on several factors including the reversal potential for GABA(A) mediated responses. Indeed, the depolarizing effect of GABA in the immature brain is demonstrated by particular physiological patterns of activity such as Giant Depolarizing Potentials in vitro (Ben-Ari et al. 1989; Khazipov et al. 2004). Yet, despite these depolarizing effects of GABA seizures do not spontaneously occur. A more positive shift in the reversal of GABA(A) mediated signals results in seizures sensitive to GABA(A) antagonists (Khalilov et al. 2003).

Thus, GABAergic control of epileptiform activity in the immature brain appears to be extremely complex with a net effect depending on variety of factors contributing to ictogenesis. Further studies are needed to determine the role of GABA in various animal models of ictogenesis and in children with epilepsy of various etiologies.
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**FIGURE LEGENDS**

FIG. 1. Hippocampal seizures are induced by repetitive intrahippocampal injection of the high-K⁺ (10 mM) / low Mg²⁺ solution in the rat in vivo. *A*: extracellular field potential recordings from CA3 pyramidal cell layer of responses to repetitive microinjections (arrows) of high-K⁺/low Mg²⁺ ACSF solution (5 µl with 10 min intervals). Note that seizure-like events are induced from the 7th application. *B*: example of ictal-like event (10th application from the panel *A*) on expanded time scale. *C*: phases of the ictal-like event: (a) initial bursting discharge, (b) tonic and
(c) clonic. D: summary plot of seizure probability as a function of the number of applications obtained from 5 rats of postnatal days P8-12.

FIG. 2. Occurrence of high-K⁺ / low Mg²⁺- induced seizures is facilitated by co-application with the GABA(A) antagonist bicuculline. A: responses evoked by four co-applications of high-K⁺/ low Mg²⁺ solution and bicuculline (arrows), recorded in CA3 pyramidal cell layer. Note that seizure-like events are generated in response to second application. B and C: example of the ictal-like event on expanded time scale and its phases. D: average plot of seizure probability obtained from 5 animals.

FIG. 3. Pharmacology of high-K⁺/ low Mg²⁺ induced seizures. Summary plot representing the effects of various GABA(A) and glutamate receptor acting drugs on the efficiency of the high-K⁺ / low Mg²⁺ to induce hippocampal electrographic seizure (n= 30 rats). Ordinates: inversed number of applications inducing seizure in 50% of cases.

FIG. 4. Effect of the GABA(A) acting drugs on high-K⁺/ low Mg²⁺ induced epileptiform activity in the hippocampal slices in vitro. A: responses evoked by application of high-K⁺/ low Mg²⁺ solution in P8 hippocampal slice, recorded in CA3 pyramidal cell layer. Parts of the ictal-like event (a, b and c) are shown below on expanded time scale. Below are shown responses to high-K⁺/ low Mg²⁺ solution in presence of 10 μM - gabazine and 10 μM – isoguvacine. Summary plots show the maximal amplitude (B) and the maximal frequency (C) of the population spikes during the epileptiform discharges.
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FIG 4. Effect of the GABA(A) acting drugs on high-K\textsuperscript{+}/ low Mg\textsuperscript{2+} induced epileptiform activity in the hippocampal slices in vitro. A: responses evoked by application of high-K\textsuperscript{+} / low Mg\textsuperscript{2+} solution in P8 hippocampal slice, recorded in CA3 pyramidal cell layer. Parts of the ictal-like event (a, b and c) are shown below on expanded time scale. Below are shown responses to high-K\textsuperscript{+}/ low Mg\textsuperscript{2+} solution in presence of 10 µM -gabazine and 10 µM isoguvacine. Summary plots show the maximal amplitude (B) and the maximal frequency (C) of the population spikes during the epileptiform discharges.