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Shunting and Hyperpolarizing GABAergic Inhibition in the High-Potassium Model of Ictogenesis in the Developing Rat Hippocampus

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ABSTRACT: Ontogenesis of GABAergic signaling may play an important role in developmental changes in seizure susceptibility in the high-potassium model of ictogenesis in vitro. The age-dependent effects of $[K^+]_o$ on the reversal potential of the GABA(A)-mediated responses and membrane potential in hippocampal slices in vitro were compared with the effect of GABA(A)-receptors antagonists and GABA(A) modulators on high-potassium induced seizures in the CA3 pyramidal layer of rat hippocampus in vivo. GABA(A) responses were depolarizing at P8–12 and hyperpolarizing at P17–21. In P8–12 rats, GABA(A) responses switch their polarity from depolarizing to hyperpolarizing upon elevation of extracellular potassium. At ~10 mM $[K^+]_o$, activation of GABA(A) receptors produced an isoelectric, purely shunting response characterized by no changes in the membrane potential but an increase in the membrane conductance. In P17–21 rats, the hyperpolarizing GABA(A) driving force progressively increased with elevation of $[K^+]_o$. In P8–12 rats in vivo, GABA(A)-receptor antagonists did not affect the occurrence of ictal discharges induced by intrahippocampal injection of 10 mM $[K^+]_o$, but significantly increased seizure duration. Diazepam and isoguvacine completely prevented seizures induced by 10 mM $[K^+]_o$. In P17–21 rats, GABA(A)-receptor antagonists strongly increased the occurrence of ictal activity induced both by 10 mM $[K^+]_o$. Taken together, these results suggest that anticonvulsive effects of GABA are because of the combination of shunting and hyperpolarizing actions of GABA. Although shunting GABA is already efficient in the young age group, a developmental increase in the hyperpolarizing GABA(A) driving force likely contributes to the increase in the GABAergic control of seizures upon maturation. © 2007 Wiley-Liss, Inc.

KEY WORDS: early seizures; GABA; CA3 pyramidal cells; gramicidin

INTRODUCTION

GABA is the main inhibitory neurotransmitter in the adult brain. GABAergic inhibition exerts powerful control of the neuronal networks and prevents paroxysmal activity. GABAergic inhibition is mainly based on two postsynaptic mechanisms: neuronal hyperpolarization and shunting. Hyperpolarization mechanisms implies that the reversal potential of the GABA(A)-mediated responses (E_{GABA}) is more negative than the resting membrane potential (V_m) and the activation of GABA receptors results in neuronal hyperpolarization that brings the membrane potential away from the action potential threshold (Bormann, 1988; Nicoll et al., 1990). The shunting mechanism is based on an increase in the membrane conductance and shunting of the depolarizing input (Qian and Sejnowski, 1990; Staley and Mody, 1992; Lu and Trussell, 2001). The shunting mechanism is inhibitory regardless of the polarity of GABAergic signals and it operates even when GABAergic signals are depolarizing.

GABAergic signaling undergoes significant changes during development. Early in development, GABA, acting via GABA(A) receptors, exerts a depolarizing action on immature neurons (Ben-Ari et al., 1989; Chen et al., 1996; Khalilov et al., 1999). The depolarizing action of GABA is because of the increased concentration of intracellular chloride ions as a result of the developmental changes in the expression and function of the transporters controlling intracellular chloride homeostasis (Rivera et al., 1999). The value of E_{GABA} in the immature neurons is more positive than the action potential threshold and activation of GABA(A) receptors excites immature neurons. Depolarizing GABA also activates voltage-gated calcium channels and potentiates the activity of NMDA channels via attenuation of their voltage-dependent magnesium block (Ben-Ari et al., 1989; Khazipov et al., 1997; Leinekugel et al., 1997; Garaschuk et al., 1998; Lamsa et al., 2000). However, E_{GABA} in immature neurons is more negative than the reversal potential of the glutamatergic responses. Therefore, depolarizing and excitatory GABA may also exert inhibitory action via shunting mechanism. Thus, GABA exerts dual—both excitatory and inhibitory—actions in the immature neurons and networks.

On the basis of the developmental changes in E_{GABA}, it can be hypothesized that depolarizing and excitatory GABA responses may be directly involved in the initiation and generation of paroxysmal events. Indeed, using the high-potassium model of ictogenesis in the hippocampal slices in vitro, it was demonstrated that during the second postnatal week, ictal activity is suppressed in a proportion of slices by GABA(A) antagonists (Dzhala

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and Staley, 2003; Khazipov et al., 2004). The NKCC1 antagonist bumetanide, which shifts depolarizing E_{GABA} to more negative values, also suppresses seizures in the young rat (Dzhala et al., 2005). Similarly, ictal activity has been reported to be potentiated by GABA(A) agonists and is insensitive to barbiturates (Dzhala and Staley, 2003). On the other hand, a number of observations indicate that during early development, depolarizing GABA also exerts anticonvulsive actions. Blockade of GABA(A) receptors induces epileptiform activities both in vitro and in vivo strongly increases the amplitude of population spikes in the high-potassium model, and aggravates seizures in several other models of ictogenesis, whereas drugs enhancing GABA(A) responses are anticonvulsants (Velisek et al., 1995; Kubova et al., 1999; Isaev et al., 2005). It has been proposed that anticonvulsive effects of GABA in the developing networks are because of the shunting mechanism and that the complexity and controversy in the results on GABAergic mechanisms in the high-potassium model are due to the dual—depolarizing and shunting—effects of GABA.

Understanding of the GABAergic mechanisms in the high-potassium model requires a consideration of multiple effects of extracellular potassium. Many elements of neuronal and network excitability including the resting membrane potential, E_{GABA}, and the resulting GABA(A) driving force (the difference between V_m and E_{GABA}) are dependent on $[K^+]_o$. However, little is known about the developmental changes in the effects of elevated $[K^+]_o$ on the V_m, E_{GABA}, and GABA(A) driving force. For the present study, we choose two age groups of rats, postnatal day (P) 8–12 and P17–21, to compare the effect of elevated $[K^+]_o$ on these parameters in vitro and the probability of inducing seizures in vivo. These ages were chosen because the second postnatal week has been shown to be a critical period of increased excitability (Jensen et al., 1991; Khazipov et al., 2004) while during the third week of postnatal development, the sensitivity of the neuronal network to epileptogenic agents substantially decreases. Using gramicidin-perforated patch recordings from CA3 pyramidal cells, we found that elevation of $[K^+]_o$ caused a positive shift of E_{GABA} and neuronal depolarization in both age groups. Neuronal depolarization was 3-fold steeper than the positive shift of E_{GABA} as a function of $[K^+]_o$. In parallel experiments in vivo, we found that GABA(A) antagonists aggravate and GABA(A) enhancing drugs (isoguvacine, diazepam) completely prevent ictal activity induced by intrahippocampal injection of 10 mM $[K^+]_o$. This suggests that isoelectric GABA(A) responses provide an anticonvulsive effect in the immature rats via a shunting mechanism. We also provide evidence that development of hyperpolarizing GABA(A) inhibition contributes to an age-dependent increase of the anticonvulsive role of GABA.

MATERIALS AND METHODS

Animals

Sprague-Dawley rat pups used throughout the study and experimental procedures were approved by the Animal Care and Use Committee at Dartmouth Medical School, and performed in accordance with the guidelines set by the National Institute of Health for the humane treatment of animals. In in vitro and in vivo experiments, we used two groups of rats P8–12 and P17–21. Injection of 10 mM $[K^+]_o$ did not induce epileptiform activity in younger rats probably due to the low synaptic connectivity between the cells in this period of life (P1–6; $n = 14$) (Isaev et al., 2005).

In Vitro Patch-Clamp Recordings

Rats were deeply anesthetized with isoflurane and decapitated. Hippocampal slices were prepared as described previously (Khazipov et al., 2004; Isaev et al., 2005). Hippocampal slices (500 μm) were cut using a Leica VT 1000S vibroslicer (Leica Microsystems, Nussloch GmbH, Germany). For recordings, slices were transferred to a chamber mounted on an Olympus BX51WI (Oberkochen, Germany) microscope with a ×40 water-immersion objective and infrared, differential interference contrast optics. The slices were continuously superfused with oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF) consisting of the following composition: 125 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 24 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, and 11 mM glucose at a rate of 2–4 ml/min at 30–32°C.

For perforated-patch recordings, we used the antibiotic ionophore-gramicidin (Sigma, St. Louis, MO) as the membrane-perforating agent to record the GABA(A)-mediated currents without influencing the intracellular Cl^- concentration (Myers and Haydon, 1972; Ulrich et al., 1987; Kyrozis and Reichling, 1995). Gramicidin was dissolved in dimethylsulfoxide (DMSO; 5–10 mg/ml) and diluted in the pipette solution to a final concentration less than 1 h before the experiment. Patch electrodes were made from borosilicate glass capillaries (GC150F-15, Clark Electromedical Instruments) and filled with a solution of the following composition (in millimolar): K-gluconate 110, KCl 25, NaCl 8, HEPES 10, and EGTA 10 (pH 7.3) that also contained 30–40 μg/ml gramicidin. The tip of the electrode was filled with the same solution but without gramicidin. Membrane potentials were corrected for liquid junction potential.

Perforated-patch recordings were made using an Axopatch 200B amplifier (Axon Instruments) from visually identified CA3 neurons in the stratum pyramidale. Pipette resistances ranged from 5 to 7 MΩ, and seal resistances were 1–10 GΩ. The progress of perforation was evaluated after the cell-attach formation by monitoring the capacitance current transient to a 5 mV step in holding potential −60 mV until the series resistance stabilized to 30–80 MΩ (after 40–60 min). Synaptic responses were evoked by electrical field stimulation (15–70 V, 50–100 μs, and 0.33 Hz) using a Grass S88 (Grass Instrument, Quincy, MA) stimulator and stimulating bipolar electrode placed in the dentate gyrus (DG) region and CA3 stratum radiatum (Davies et al., 1990).

For voltage-clamp and current-clamp recordings, slices were superfused with ACSF in the presence of the GABA(B)-receptor antagonist CGP 35 348 (10 μM), and the glutamate receptor antagonists D-2-amino-5-phosphonovalerate (D-APV, 50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) for

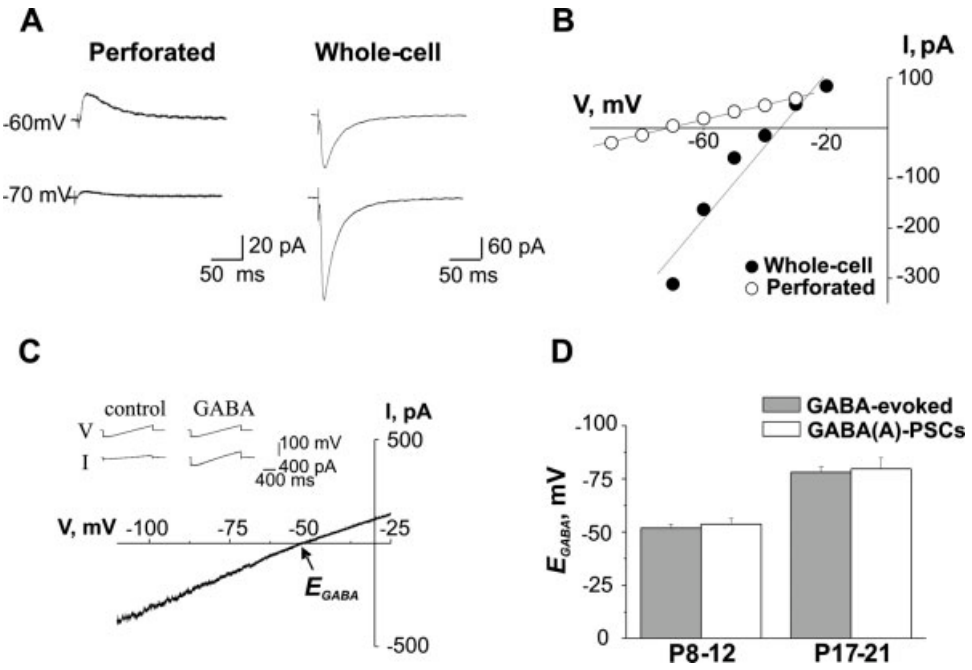

FIGURE 1. Gramicidin perforated-patch recordings of the GABA(A) mediated responses in CA3 pyramidal cells in the hippocampal slice. A. GABA(A)-postsynaptic currents (PSCs) evoked by electrical stimulation of the DG region obtained with gramicidin perforated patch recording (left) and after subsequent conversion to a whole-cell configuration (right) at holding potentials of −60 and −70 mV. Recordings from P17 CA3 pyramidal neuron in the presence of 10 μM CNQX, 50 μM D-APV, and 10 μM CGP 35 348. B. Corresponding current-voltage relationships of the GABA(A)-PSCs in gramicidin perforated-patch (open circles) and whole-cell (filled circles) configurations. With the perforated patch, the E_{GABA} was ~−74 mV, whereas in whole-cell mode, the E_{GABA} shifted to −36 mV, which is close to the value of the Nernst equilibrium potential for given extra-/intracellular concentrations. C. Current-voltage relationships of the responses evoked in P9 CA3 pyramidal cell by application of 50-μM GABA. (Inset) Example of ramp currents recorded in perforated-patch mode in control or 50-μM GABA added ACSF. Currents were recorded in response to a voltage ramp from −110 to −20 mV from a holding potential of −60 mV. Gramicidin perforated patch recordings were made in the presence of 10 μM CGP 35 348, 10 μM $CdCl_2$, and 1 μM TTX. D. Reversal potentials of the responses evoked by GABA application (gray) and of the GABA(A)-PSCs in P8–12 and P17–21 age groups.

pharmacological isolation of monosynaptic inhibitory responses. Bath application of 15 μM bicuculline resulted in a complete reversible block of any detectable activity (data not shown). After recordings in gramicidin-perforated patch configuration, the conventional whole-cell configuration was established. This resulted in a shift of the E_{GABA} towards −36 mV that corresponds to the equilibrium potential for given inside and outside concentrations of Cl^- calculated by the Nernst equation (Figs. 1A,B). These findings demonstrated that the recording evoked postsynaptic currents (PSCs) are GABA-mediated Cl^- currents. To determine E_{GABA} for these experiments, 50–60 GABA(A)-PSCs were recorded and averaged for every holding potential.

The high series resistance is one of the technical limitations in the gramicidin-perforated patch-clamp studies (Ulrich et al., 1987; Vale and Sanes, 2000; De Jeu and Pennartz, 2002). To assure that a high series resistance did not substantially influence on results, we calculated the mean voltage error according to the equation:

$$V_{err} = I_h \times R_s$$

in which V_{err} is the error voltage, I_h is the holding current, and R_s is the series resistance (Ulrich et al., 1987; Armstrong and Gilly, 1992), and the mean voltage error in our studies was estimated to lie in the range of 0.5–3.0 mV. This voltage error is generally considered acceptable and we therefore did not correct our data. We also performed additional experiments to estimate the E_{GABA} of GABA-evoked (somatic) currents from CA3 pyramidal neurons and compared these results with the E_{GABA} estimated for the pharmacologically isolated GABA(A)-PSCs induced by electrical stimulation of the hilus and CA3 stratum radiatum (Davies et al., 1990). Determination of E_{GABA} for GABA-evoked currents were made in control ACSF (3.5 mM $[K^+]_o$) and studied using a depolarizing voltage ramp protocol (Figs. 1C,D). For these experiments, we added TTX (1 μM) and $CdCl_2$ (10 μM) to block the voltage-activated Na^+ and Ca^{2+} currents, respectively, and 10 μM CGP 54845A to block the $GABA_B$ receptor-mediated currents. Membrane voltage (V_h = −60 mV) was first stepped to −110 mV for 200 μs and then ramped from −110 to −20 mV in 1,400 μs in control ACSF. Then, GABA was puffed onto the cell body (50 μM) through a micropipette and recording were repeated again.

Experiments investigating the influence of elevated concentrations $[K^+]_o$ on E_{GABA} and V_m were performed and compared on the same cell. After recording of current-voltage relationship of GABA(A)-PSCs in control ACSF (3.5 mM $[K^+]_o$), we changed the applied solution to 5 mM $[K^+]_o$ then 10 mM $[K^+]$

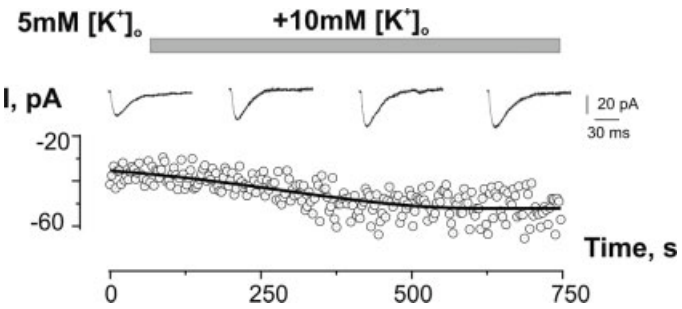

FIGURE 2. The effect of increasing extracellular potassium concentration on the eIPSCs. The plot represents time course of changing of amplitude of eIPSCs (open circles) recorded from the CA3 pyramidal neuron in perforated-patch mode after increasing $[K^+]_o$ from 5 to 10 mM at P9. Recordings were made every 3 s at holding potential −60 mV (Inset). Example traces in corresponding time. The solid line shows that after 10 min of application 10 mM $[K^+]_o$ the averaged amplitude of eIPSCs stabilizes.

ACSF. The waiting time until recorded GABA(A)-PSCs stabilized was 10–15 min (Fig. 2). This approach was applied because the membrane potential changes almost immediately after $[K^+]_o$ is changed, whereas changes in E_{GABA} depend on $[Cl^-]_i$. As the $[K^+]_o$ rises, $[Cl^-]_i$ will gradually rise inside the cell depending on activity of the cation chloride cotransporters. Previous studies measuring the kinetics of the changes in E_{GABA} as a function of $[K^+]_o$ have demonstrated that the equilibrium is attained within 10 min (DeFazio et al., 2000; Kakazu et al., 2000). We therefore waited for 10–15 min to assure that the $[Cl^-]_i$ reached a new steady-state level. The average membrane potential for every given $[K^+]_o$ was determined as a mean value of the membrane potential obtained during a recording of $\geq$2 min in current-clamp mode with null current.

In Vivo Extracellular Recordings

The surgical procedure was described in detail previously (Isaev et al., 2005). Briefly, the application cannula (0.2 mm diameter, Plastic One, Roanoke, VA) with an inserted wire electrode (0.02 mm diameter; California Fine Wire, Grover Beach, CA) was implanted into the hippocampus under deep anesthesia with isoflurane in an O_2 carrier (induction 4%, maintenance 2%) using an agent-specific vaporizer (Isotec 3, Ohmeda Medical System). For general analgesia, buprenex (0.01–0.02 mg/kg buprenorphine hydrochloride) was administered subcutaneously. The application cannula with recording electrode was positioned into the CA3 pyramidal cell layer under stereotaxic and electrophysiological guidance (2.0–2.5 mm caudal to bregma; 2.0–2.5 mm from midline; depth 2.7–3.1 mm). A reference electrode was implanted into the cerebellum. After surgery, the rat pup was allowed to recover from anesthesia for 10–15 min. Electrophysiological data were then recorded uninterrupted for 60–120 min.

Drugs were dissolved in the ACSF and applied locally through the stainless-steel cannula using microsyringes. Application was made by brief repetitive microinjections of 5-μl volumes every 5 min for up to 10 times. Infusion of ACSF did not lead to changes in neuronal activity. In the experiments with high-potassium ACSF, 10 mM NaCl was replaced with equivalent concentrations of KCl. Concentration of the infused drugs is unknown; however, similar concentration profile of the high-potassium-induced epileptiform activity in vitro and in vivo suggests that concentration of the infused drugs in a vicinity of the injection site attains the levels in the infusion solution.

Extracellular recordings were performed using a differential amplifier (A-M Systems, Carlsborg, WA) (bandpass 0.1–2 kHz; × 1,000). After the recordings, the rats were deeply anesthetized and decapitated. Sagittal 300-μm slices were cut using the vibroslicer and the position of cannula/electrode was verified under light microscopic examination.

Interictal activity was defined as brief (80–200 μs) bursts of high amplitude spikes in the EEG that occurred on a background of otherwise normal activity. Ictal activity consisted of sustained rhythmic spikes (McCormick and Contreras, 2001).

Data Acquisition and Analysis

Recordings were digitized (10 kHz) online with an analogue-to-digital converter (Digidata 1322A; Axon Instruments) and analyzed off-line with the Clampfit (Axon Instruments), Mini Analysis (version 5.5; Synaptosoft, Decatur, GA), and Origin 5.0 (Microcal Software, Northampton, MA). Theoretical equilibrium potentials for Cl^- were calculated with the use of the Nernst equation. Linear regression was used to fit the voltage dependence of GABA(A)-PSCs and the interpolated intercept of the best fit line with the abscissa was taken as the E_{GABA} of GABA(A)-PSCs. For GABA-evoked currents (somatic currents), E_{GABA} was defined as the value of the potential at the point of intersection of the abscissa with the line obtained after subtraction of the ramp line recorded in control ACSF from the ramp line recorded in GABA-added ACSF. The driving force for the GABA responses was indicated as the difference between the absolute values of V_m and E_{GABA}. Group measures were expressed as means ± SE; error bars also indicate SE. The statistical significance of differences was assessed with the Student's t test. The level of significance was set at $P < 0.05$.

Chemicals

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphonovalerate (D-APV), CGP 35 348, bicuculline, and gabazine were obtained from Tocris (Ellisville, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

We first explored the effect of elevated $[K^+]_o$ on the E_{GABA} and V_m in two age groups of rats (P8–12 and P17–21) using gramicidin-perforated patch recordings that do not affect $[Cl^-]_i$ or the reversal potential of the GABA(A)-mediated responses (Abe et al., 1994; Kyrozis and Reichling, 1995). Experiments were performed from visually identified CA3 pyramidal cells in hippocampal slices in vitro. Two types of GABA(A) receptor-mediated responses were studied and compared: pharmacologically isolated GABA(A)-mediated PSCs (GABA(A)-PSCs) and responses to local application of GABA. GABA(A)-PSCs were evoked by electrical stimulation of

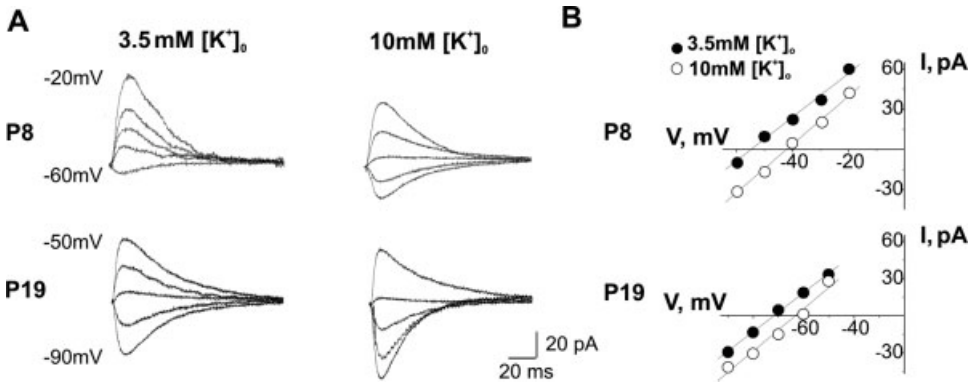

FIGURE 3. Age-dependence of the effect of $[K^+]_o$ on the GABA(A) reversal potential. A. GABA(A)-PSCs recorded from CA3 pyramidal neurons in the P8 and P19 hippocampal slices at 3.5 (left) and 10 mM $[K^+]_o$ (right) at different holding potentials. B. Corresponding I–V relationships of the GABA(A)-PSCs in the presence of 3.5 and 10 mM $[K^+]_o$ at P8 and P19. Note that increases in $[K^+]_o$ induces positive shift of E_{GABA} in both age groups.

the DG region and CA3 stratum radiatum in the presence of the ionotropic glutamate receptors antagonists CNQX (10 μM) and D-APV (50 μM) and GABA(B)-receptor antagonist CGP 35348 (10 μM) (Fig. 1A). The responses evoked by local somatic application of GABA (50 μM) were recorded in the presence of TTX (1 μM) to block sodium action potentials, and $CdCl_2$ (10 μM) to block voltage-gated calcium channels. CGP 35348 (10 μM) was used to block GABA(B)-mediated responses (Fig. 1C). Both types of responses were reversibly blocked by the GABA(A) receptor antagonist bicuculline (15 μM; not shown). Study of the current-voltage relationships of the GABA(A)-PSCs and the responses evoked by GABA application revealed that the E_{GABA} for GABA-evoked currents in control ACSF (3.5 mM $[K^+]_o$) at the age P8–12 and P17–21 were not significantly different from GABA(A)-PSCs induced by electrical field stimulation (-52.1 ± 1.9 mV ($n = 15$) vs. -53.8 ± 2.8 mV ($n = 17$) for P8–12, -78.2 ± 2.6 ($n = 17$) vs. -79.9 ± 5.2 mV ($n = 19$) for P17–21, respectively, $P > 0.05$) (Fig. 1D). The values of E_{GABA} are in agreement to report reversal potential of the GABA(A)-mediated responses at comparable ages (Swann et al., 1989; Zhang et al., 1990; Luhmann and Prince, 1991; Rovira and Ben-Ari, 1993; Psarropoulou and Descombes, 1999; Tyzio et al., 2003).

Elevation of $[K^+]_o$ from 3.5 to 5 mM and 10 mM resulted in the progressive positive shift of E_{GABA} in both age groups (Figs. 3

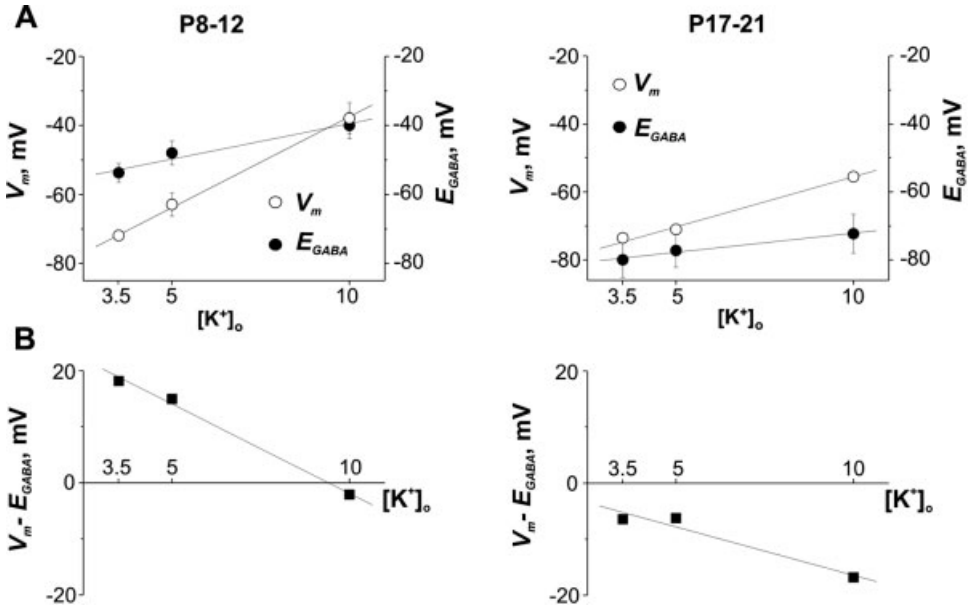

FIGURE 4. Age-dependence of the effect of $[K^+]_o$ on the membrane potential, GABA(A) reversal potential and GABA(A) driving force. A. Dependence of the membrane potential V_m (open circles) and E_{GABA} (filled circles) on $[K^+]_o$ at P8–12 (left) and P17–21 (right) (means ± SEM). Note, that neuronal depolarization induced by a rise in $[K^+]_o$ is steeper than the positive shift of the GABA(A) reversal potential in both age groups. B. Dependence of the GABA(A) driving force (the difference between absolute values of V_m and E_{GABA}) on $[K^+]_o$ at P8–12 (left) and P17–21 (right).

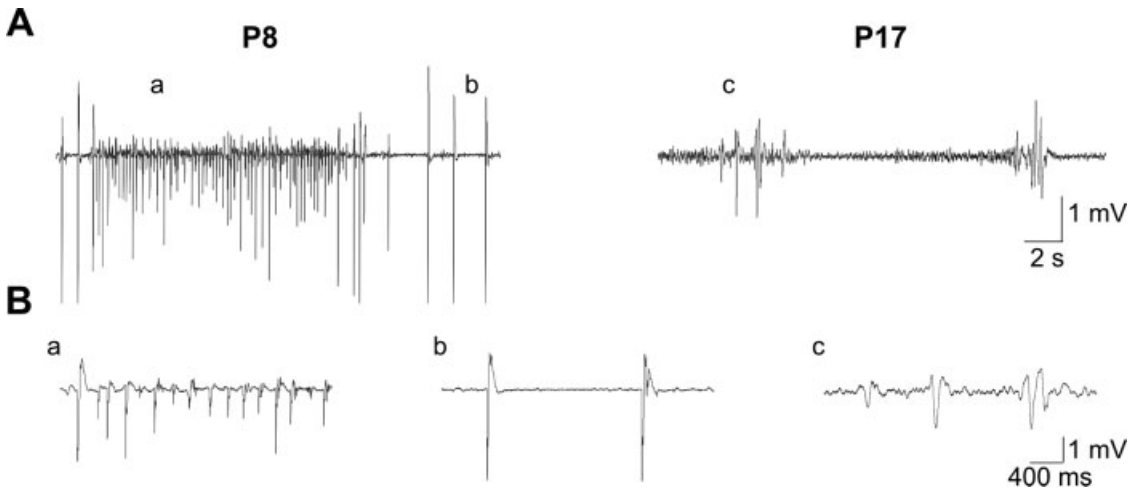

FIGURE 5. Age-dependence in the occurrence of hippocampal ictal and interictal activity induced by intrahippocampal injection of high-potassium ACSF in vivo. Extracellular field potential recordings from the CA3 pyramidal cell layer in the presence of 10 mM $[K^+]_o$ in vivo. A. At P8, application of high-potassium ACSF led to ictal activity, whereas at P17, only interictal activity was recorded. B. Parts of the traces from A on an expanded time scale: (a) ictal discharges at P8, (b and c) interictal activity at P8 and P17, respectively.

and 4). In parallel, elevation of $[K^+]_o$ caused neuronal depolarization. However, the dependence of E_m on $[K^+]_o$ was nearly 3-fold steeper than that of E_{GABA}. This result correlates well with previous data obtained in other laboratories (Thomson and Gahwiler, 1989; DeFazio et al., 2000; Kakazu et al., 2000). As a result, the GABA(A) driving force, the difference between E_{GABA} and E_m, switched polarity from positive (depolarizing) to negative (hyperpolarizing) upon elevation of $[K^+]_o$ in the young age group (P8–12), whereas only an increase in the negative GABA(A) driving force was observed in P17–21 cells. Figure 4 shows results of group analysis on the dependence of V_m, E_{GABA}, and GABA(A) driving force on $[K^+]_o$ in P8–12 ($n = 17$) and P17–21 cells ($n = 19$). There was a remarkable age-dependent difference in the effect of $[K^+]_o$ on GABA(A) driving force between the two age groups. In P8–12 rats, elevation of $[K^+]_o$ caused a switch in the polarity of the GABA(A)-PSCs from depolarizing to hyperpolarizing, while in older (P17–21) rats, GABA remained hyperpolarizing at all concentrations of $[K^+]_o$ studied. In P8–12 neurons, activation of GABA(A) receptors at 9.5 mM $[K^+]_o$ produced a purely isoelectric shunting response with an increase in the membrane conductance without a significant change in the membrane potential.

In the next series of experiments, we studied GABAergic modulation of hippocampal seizures as a function of postnatal age using the high-potassium model of ictogenesis in vivo in two age group P8–12 and P17–21. Hippocampal seizures were evoked by intrahippocampal infusion of the high-potassium solution and were monitored by an extracellular electrode placed in CA3 pyramidal cell layer. Focal infusion of 10 mM $[K^+]_o$ ACSF into the CA3 pyramidal cell layer of the hippocampus generated ictal activity in 3 of 9 (33%) animals studied at the ages of P8–12. Interictal activity was observed in the remaining six animals in this age group. At P17–21, 10 mM $[K^+]_o$ evoked interictal activity in two of six animals but no ictal activity was observed ($n = 6$) (Fig. 5).

We further studied the age-dependence in the effect of the GABA(A)-antagonist on occurrence of ictal activity. The GABA(A)-antagonists bicuculline (15 μM) or gabazine (10 μM) were coapplied together with 10 mM $[K^+]_o$ and the incidence of ictal activity was compared to the injection of 10 mM $[K^+]_o$ alone. At P8–12, ictal activity was obtained in three of eight rats (37%) treated with 10 mM $[K^+]_o$ and bicuculline ($n = 5$) or gabazine ($n = 3$). This value did not significantly differ from seizure probability in response to injection of 10 mM $[K^+]_o$ alone (33%) (Fig. 7). More detailed analysis of the epileptiform activity revealed that frequency and amplitude of ictal activity evoked by 10 mM $[K^+]_o$ was not significantly different from those evoked by 10 mM $[K^+]_o$ + GABA(A) antagonist ($P = 0.05$; (Fig. 6). There were not any changes in the number of applications needed for triggering of ictal discharges (not shown). However, blockade of GABA(A) receptors increased nearly 5-fold the duration of ictal discharges (from 11.7 ± 2.8 to 58.3 ± 6.0 s) (Fig. 7C). Coapplication of the GABA(A) receptor agonist isoguvacine (10 μM; $n = 6$) and diazepam, a positive allosteric modulator of the GABA(A) receptors (5 μM; $n = 6$) with 10 mM $[K^+]_o$ completely prevented seizures (not shown).

Although 10 mM $[K^+]_o$ did not cause ictal activity in P17–21 rats ($n = 6$), coapplication of the GABA(A)-antagonists together with 10 mM $[K^+]_o$ caused ictal events in all cases studied (Fig. 7; $n = 4$).

DISCUSSION

The principal observations of the present study can be summarized as follows: (i) elevation of extracellular potassium causes a depolarizing to hyperpolarizing switch of the GABA(A) mediated responses in CA3 pyramidal cells and at ~10 mM $[K^+]_o$, activation of GABA(A) receptors evokes purely isoelectric shunting

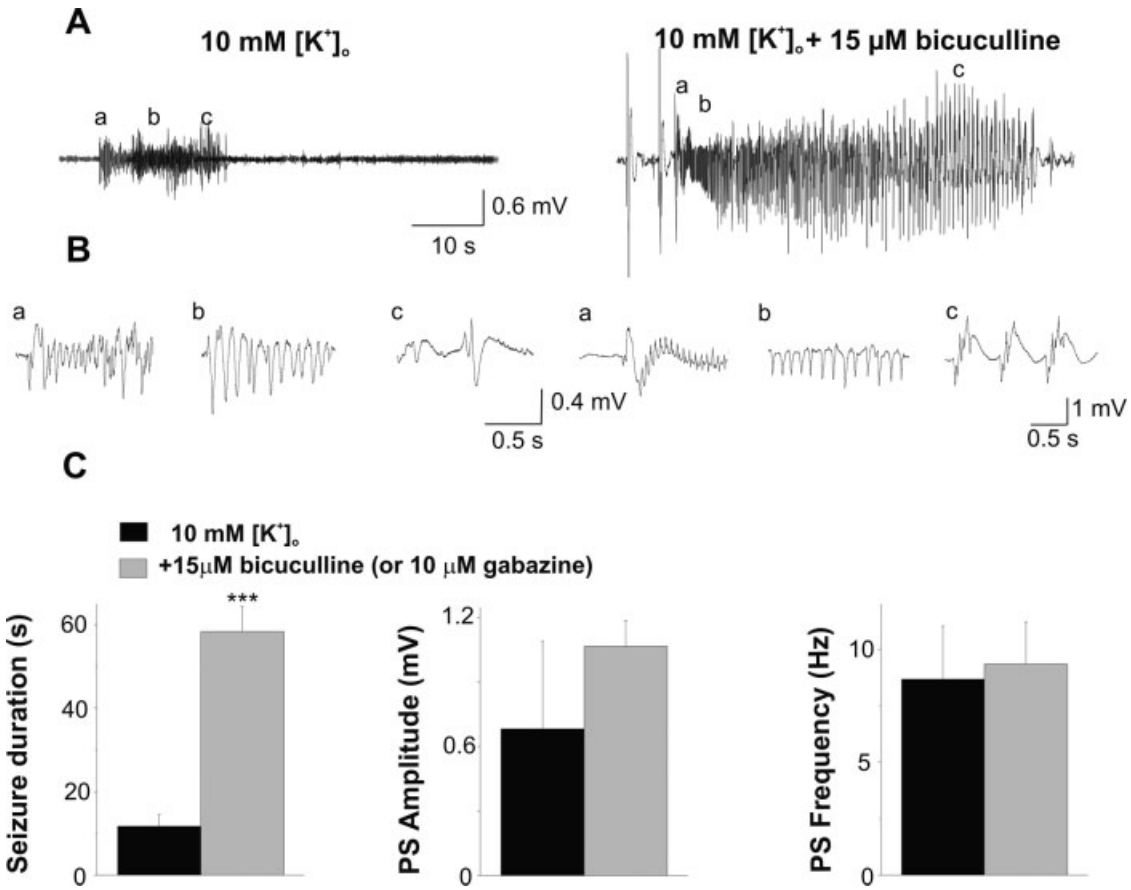

FIGURE 6. Effect of the GABA(A) receptor antagonists on the ictal activity induced by high potassium in vivo. A. Examples of ictal activity induced by repetitive intrahippocampal injections of 10 mM $[K^+]_o$ containing ACSF in control conditions (left panel) and in the presence of 15 μM bicuculline (right panel) presented in the same scale. Recordings were made from CA3 pyramidal cell layer of hippocampus. B. The phases of ictal activity from A are shown on expanded time scale: (a) initial bursting discharges, (b) tonic-like and (c) clonic-like activity. C. Summary plots show the duration of ictal activity, the maximal amplitude and the maximal frequency of the population spikes during the epileptiform discharges induced by injection of 10 mM $[K^+]_o$ in the absence (black columns, $n = 3$) or presence of 15 μM bicuculline (or 10 μM gabazine) (gray columns, $n = 3$). Pooled data from P8–12 rats. Means are ±SD error of mean (SEM).

responses during the second postnatal week; (ii) the GABA(A) antagonists prolong and the GABA(A) enhancing drugs prevent ictal activity induced by 10 mM $[K^+]_o$ in vivo suggesting an anticonvulsive GABA(A) shunting mechanism; (iii) hyperpolarizing GABA(A) mediated responses observed in the older animals provide powerful anticonvulsive action. Taken together, these results suggest that in the high-potassium model of ictogenesis, isoelectric GABA exerts anticonvulsive actions in the young (P8–12) animals probably via shunting mechanism and a more efficient anticonvulsive action in older (P17–21) animals probably via a combination of both shunting and hyperpolarizing mechanisms.

As observed in the present study, the dependence of E_{GABA} on $[K^+]_o$ is in agreement with the results of previous findings that have suggested that a rise in $[K^+]_o$ results in inhibition or even reversal of the chloride extruder KCC2 and an increase in $[Cl^-]_i$ (Thompson and Gahwiler, 1989; Jensen et al., 1993; Payne et al., 1996; Kaila et al., 1997; DeFazio et al., 2000; Kakazu et al., 2000).

A rise in $[K^+]_o$ also increases the firing of interneurons and causes a massive release of GABA producing an activity-dependent depolarizing shift in E_{GABA} via collapse in the chloride gradient and an increase in the contribution of bicarbonate to GABA(A) mediated conductance (Staley et al., 1995) similar to findings in the crayfish muscle (Kaila and Voipio, 1987). However, the bicarbonate-mediated depolarizing mechanism does not operate until P12, when intraneuronal carbonic anhydrase emerges in the hippocampal pyramidal neurons (Rivera et al., 2005). It was also demonstrated that in adult neurons, a depolarizing shift of E_{GABA} caused by an increase in $[K^+]_o$ does not exceed neuronal depolarization and therefore the GABA(A) driving force remains hyperpolarizing (Kaila et al., 1997; Smirnov et al., 1999). In agreement with these observations, we also found that GABAergic responses remain hyperpolarizing in the older rats upon a rise in $[K^+]_o$. In the young neurons from P8–12 rats, the positive direction and magnitude of E_{GABA} change was similar to that observed in older neurons. Despite $[Cl^-]_i$ in P8–12, neurons are supported by NKCC1 and KCC2, transport through NKCC1 is relatively insensitive to manipulations of $[K^+]_o$ (DeFazio et al., 2000). However, because neuronal depolarization was 3-fold steeper than

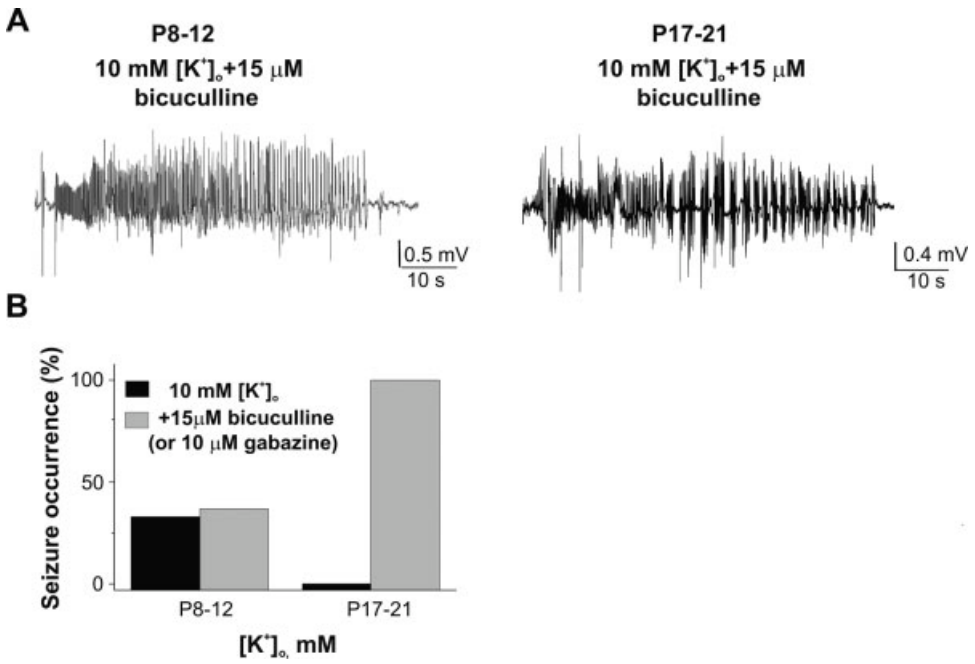

FIGURE 7. Effect of the GABA(A) receptor antagonists on occurrence of the hippocampal ictal activity induced by different $[K^+]_o$ at P8–12 and P17–21 in vivo. A. Examples of the hippocampal ictal activity induced by intrahippocampal injection of 10 mM $[K^+]_o$ together with the GABA(A) antagonists bicuculline or gabazine at P8–10 (left panels) and P17–21 (right panels). B. Summary of the effect of blockade GABA(A)-receptors on seizure occurrence at different $[K^+]_o$ at P8–10 and P17–21. Columns represent percentage of seizure occurrence in the absence (black) or presence (gray) of gabazine/bicuculline. Pooled data from 27 rats.

E_{GABA} as a function of $[K^+]_o$, and because E_{GABA} was depolarizing at physiological $[K^+]_o$, the depolarizing to hyperpolarizing switch in the GABA(A) driving force occurred upon an increase in $[K^+]_o$ in P8–12 neurons. This phenomena has been observed in several laboratories where researchers have found variations in the increase of $[Cl^-]_i$ in response to increasing $[K^+]_o$ (Thomson and Gahwiler, 1989; DeFazio et al., 2000; Kakazu et al., 2000). Kakazu et al. (2000) showed that the increase of $[Cl^-]_i$ as a function of the increase in $[K^+]_o$ corresponded to a rate of 1:2. The mechanism of such a distribution is unclear and could not be explained solely by the operation of the electrically neutral Cl^- cation cotransporters. As a result, with increasing $[K^+]_o$ the difference between depolarizing E_{GABA} and V_m is reduced and at a certain point activation of GABA receptors should have only a shunting effect. We found that in P8–12 rats, GABA(A) mediated responses switched their action from depolarizing to hyperpolarizing upon elevation of $[K^+]_o$ at 9.5 mM. At this isoelectric point of the switch, activation of GABA(A) receptors did not affect the membrane potential but only increased membrane conductance, producing a purely shunting effect.

Previous studies have shown both proconvulsive and anticonvulsive roles for GABA in the immature hippocampus (Khalilov et al., 1999; Wells et al., 2000; Khazipov et al., 2004; Dzhala et al., 2005; Isaev et al., 2005). In the high-potassium model in vitro, it was shown that during the second postnatal week, blockade of GABA(A) receptors suppresses ictal activity transforming it to interictal activity composed of large amplitude population spikes (Dzhala and Staley, 2003). In a similar study, suppression of ictal discharges by the GABA(A) antagonists was observed in one-third of slices while in the remaining two-thirds of slices, GABA(A) antagonists only reduced the frequency of ictal discharges (Khazipov et al., 2004). Interestingly, in the majority of slices that generated only interictal activity under elevated potassium conditions, GABA(A) antagonists caused a single episode of ictal activity that was followed by large amplitude clonic-like recurrent events. Blockade of GABA(A) receptors at physiological $[K^+]_o$ (at which depolarizing GABA(A) driving force is maximal) blocks the physiological pattern of giant depolarizing potentials and induces interictal or ictal activity during the second postnatal week (Swann and Brady, 1984; Khalilov et al., 1997, 1999; Lamsa et al., 2000). In the present study, we found that blockade of GABA(A) receptors does not modify the occurrence but strongly prolonged the duration of ictal discharges induced by 10 mM $[K^+]_o$. We also found that the GABA(A) agonist isoguvacine and the positive GABA(A) modulator diazepam completely prevent high potassium-induced seizures, similar to our previous findings using high-potassium/low magnesium model (Isaev et al., 2005). These findings are in agreement with the results obtained in vitro, in which 10–20 μM isoguvacine efficiently suppressed the ictal- and interictal-like activities induced by high-potassium (Dzhala and Staley, 2003).

We found that GABA may rapidly change its action from depolarizing to hyperpolarizing within a window of changes in $[K^+]_o$ occurring during seizures (Fig. 4). According to the results of present work and in keeping with anticonvulsive effects of the GABA(A) agonists at physiological $[K^+]_o$ (Swann and Brady, 1984; Khazipov et al., 1997; Khalilov et al., 1999; Lamsa et al., 2000; Wells et al., 2000; Isaev et al., 2005), depolarizing and

isoelectric GABA may be anticonvulsive via a shunting mechanism. On the other hand, depolarizing GABA may be proconvulsive when the excitatory action of GABA overrides the shunting effect (Dzhala and Staley, 2003; Dzhala et al., 2005). Thus, it is likely that the apparent discrepancies in the results on GABAergic modulation of seizures reflect complex pro- and anticonvulsive GABAergic mechanisms that reflect the dual—excitatory and inhibitory—nature of GABAergic signaling in the immature networks.

Although anticonvulsive GABAergic actions are present both in young and older animals, there is a developmental change in the GABAergic control of seizures. GABAergic control of the network is achieved via two mechanisms: shunting, which does not depend on the polarity of the GABA(A) mediated responses, and its hyperpolarizing effect. It is suggested that the shunting mechanism is already present during the second-postnatal week and depolarizing or isoelectric GABA(A) responses produce a shunt of the glutamatergic excitatory PSCs in the recurrent collateral synapses that are playing a principal role in synchronizing paroxysmal discharges. However, GABAergic inhibition of network activity is weaker at these developmental stages because hyperpolarizing inhibition is absent or is weaker than in the adult animals. Together with other developmental changes in neuronal, synaptic and network properties (Swann and Hablitz, 2000; Holmes et al., 2002), the lack of hyperpolarizing inhibition may well contribute to the increased seizure susceptibility during the critical developmental period of hyperexcitability. This is supported by the findings that blockade of NKCC1 by bumetanide that renders GABA with hyperpolarizing action, suppresses high-potassium induced seizures in vitro, and kainate-induced seizures in vivo (Dzhala et al., 2005).

In summary, depolarizing GABA responses can be inhibitory or excitatory, and epileptiform activities may be based both on excitatory and inhibitory GABAergic mechanisms. Complex pro- and anticonvulsive actions of GABA discussed here in the context of developmental changes in the high potassium model further emphasize the complexity of GABAergic mechanisms during seizures.

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