Published in final edited form as:

Eur J Neurosci. 2010 April ; 31(8): 1446–1455. doi:10.1111/j.1460-9568.2010.07179.x.

RECURRENT NEONATAL SEIZURES RESULT IN LONG-TERM INCREASE OF NEURONAL NETWORK EXCITABILITY IN THE RAT NEOCORTEX

Elena Isaeva^{1,2}, Dmytro Isaev^{1,2}, Alina Savrasova², Rustem Khazipov³, and Gregory L. Holmes¹

¹ Department of Neurology, Neuroscience Center at Dartmouth, Dartmouth Medical School, Lebanon, New Hampshire

² Bogomoletz Institute of Physiology, Kiev, Ukraine

³ INMED, INSERM-U29, Marseille, France

Abstract

Neonatal seizures are associated with a high likelihood of adverse neurological outcomes, including mental retardation, behavioral disorders, and epilepsy. Early seizures typically involve the neocortex, and post-neonatal epilepsy is often of neocortical origin. However, our understanding of the consequences of neonatal seizures for neocortical function is limited. In the present study, we show that neonatal seizures induced by flurothyl result in markedly enhanced susceptibility of the neocortex to seizure-like activity. This change occurs in young rats studied weeks after the last induced seizure and in adult rats studied months after the initial seizures. Neonatal seizures resulted in reductions in the amplitude of spontaneous inhibitory postsynaptic currents and the frequency of miniature inhibitory postsynaptic currents, and significant increases in the amplitude and frequency of spontaneous excitatory postsynaptic currents (sEPSCs) and in the frequency of miniature excitatory postsynaptic currents (mEPSCs) in pyramidal cells of layer 2/3 of the somatosensory cortex. The selective N-methyl-d-aspartate (NMDA) receptor antagonist d-2-amino-5-phosphonopentanoate eliminated the differences in amplitude and frequency of sEPSCs and mEPSCs in the control and flurothyl groups, suggesting that NMDA receptors contribute significantly to the enhanced excitability seen in slices from rats that experienced recurrent neonatal seizures. Taken together, our results suggest that recurrent seizures in infancy result in a persistent enhancement of neocortical excitability.

Keywords

early seizures; GABA; NMDA; somatosensory cortex

Introduction

Children during the first months of life are at particularly high risk for seizures, with the largest number of new-onset seizure disorders occurring during this time (Hauser et al., 1995). Most neonates with seizures have multiple seizures over the course of a few days (Clancy & Legido, 1987; Clancy et al., 1988). In addition to a high risk for seizures, there is

Authors for correspondence: Gregory L. Holmes, MD, Department of Neurology, Dartmouth Medical School, Dartmouth-Hitchcock Medical School, One Medical Center Drive, Lebanon, NH 03756, Gregory.L.Holmes@Dartmouth.Edu. Elena Isaeva, PhD, Department of General Physiology of Nervous System, Bogomoletz Institute of Physiology, Bogomoletz str. 4, Kiev, 01024, Ukraine, olena.isaeva@gmail.com.

evidence that neonatal seizures are associated with a considerable risk of long-term sequelae, including post-neonatal epilepsy, behavioral problems, attention deficit disorder, and mental retardation (Legido et al., 1991; McBride et al., 2000; Brunquell et al., 2002; Ronen et al., 2007; Glass et al., 2009). Animal studies over the last two decades have shown that neonatal seizures result in considerable morphological and physiological changes in the brain that persist into adulthood. These seizure-induced changes include synaptic reorganization of the axons and terminals of the dentate granule cells (Holmes et al., 1998; Huang et al., 1999), decreases in neurogenesis (McCabe et al., 2001), alterations in glutamate (Sanchez et al., 2001; Sogawa et al., 2001; Bo et al., 2004; Cornejo et al., 2007) and c-aminobutyric acid (GABA) (Ni et al., 2004) receptors, reductions in GABA synaptic transmission (Isaeva et al., 2006), decreases in excitatory amino acid carrier 1 (Zhang et al., 2004), impaired single-cell firing (Karnam et al., 2009b), and deficits in visual spatial memory (Holmes et al., 1998; Huang et al., 1999; Chang et al., 2003; Karnam et al., 2009a,b) and auditory discrimination (Neill et al., 1996). Recurrent neonatal seizures result in reduced spike frequency adaptation and after hyperpolarizing potential following a spike train in the CA1 region and long-term selective impairment in GABAergic neurotransmission in the CA3 region of the hippocampus (Villeneuve et al., 2000; Isaeva et al., 2006), changes that probably contribute to the enhanced seizure susceptibility seen following neonatal seizures. However, these studies evaluated seizure-induced changes in hippocampal excitability, and provide no information on neocortical changes following neonatal seizures. Evaluating the effect of neonatal seizures on the neocortex is important, because neonatal seizures in humans typically involve the neocortex (Duchowny & Harvey, 1996; Acharya et al., 1997; Mizrahi, 1999; Mizrahi & Clancy, 2000), and are more likely to lead to epilepsy originating in the neocortex than in the hippocampus (Ronen et al., 2007). In this study, we used the rat to gain insights into the effects of neonatal seizures on long-term neocortical function. Following flurothyl-induced repetitive seizures in young rats, we used extracellular and patch-clamp recordings in neocortical pyramidal neurons to show that neonatal seizures have long-lasting effects on the susceptibility of the somatosensory cortex to seizures and on GABAergic and glutamatergic signaling in neocortical pyramidal neurons.

Materials and methods

Animals

All experiments were performed in accordance with the guidelines set by the National Institute of Health and Dartmouth Medical School for the humane treatment of animals. Sprague-Dawley rats ($n = 42$) were subjected to 58–60 flurothyl-induced seizures from postnatal day (P)1 to P10 (five or six seizures per day), using previously described methods from our laboratory (Huang et al., 1999; Karnam et al., 2009b). Control rats ($n = 40$) were handled and treated in the same manner but not exposed to flurothyl.

Slice preparation

Brain slices were prepared from P20–P30 and P60–P80 rats. The rats were deeply anesthetized by inhalation of isoflurane, and decapitated. Brains were quickly removed and placed in a chilled (0–5°C) solution of the following composition: 250 mm sucrose, 2 mm KCl, 0.5 mm CaCl_2 , 7 mm MgCl_2 , 26 mm NaHCO_3 , 1.2 mm NaH_2PO_4 , and 11 mm glucose (pH 7.4). Slices were cut using a Leica 1000S vibroslicer (Leica Microsystems, Nussloch GmbH, Germany) and transferred to an incubation chamber where they rested submerged in oxygenated (95% O_2 – 5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition: 126 mm NaCl, 3.5 mm KCl, 2 mm CaCl_2 , 1.3 mm MgCl_2 , 25 mm NaHCO_3 , 1.2 mm NaH_2PO_4 , and 11 mm glucose (pH 7.3–7.4). Slices were allowed to incubate for at least one hour before recordings were made.

Electrophysiology

Following incubation, a single slice was transferred to a recording chamber (Warner Instrument Corp., Hamden, CT, USA) mounted on an Olympus BX51WI (Japan) and perfused with oxygenated ACSF at 2 ml/min. Cell types were identified and accessed using infrared differential interference contrast (IR-DIC) optics with a X40 water immersion objective.

All patch-clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments). sIPSCs and sEPSCs were recorded from pyramidal neurons in layer (L) 2/3 of somatosensory cortex using voltage-clamp technique in a whole-cell configuration. Patch electrodes were made from borosilicate glass capillaries (GC150F-15, Clark Electromedical Instruments) and filled with a solution of the following composition (in mM): Cs-gluconate 117.5, CsCl 17.5, NaCl 8, HEPES 10, EGTA 10, Na₃GTP 0.2, and MgATP 2 (pH 7.3). Pipette resistances were ranged from 3–5 M Ω , and seal resistances were 5–10 G Ω . The series resistances were compensated on 80–90% (lag 20 μ s).

sIPSCs were recorded at a holding potential 0 mV (reversal potential for sEPSCs). sEPSCs were recorded at a holding potential –80 mV in ACSF containing 10 μ M gabazine, a selective blocker of the GABA_A receptor. Because increasing temperature strongly increases the frequency of spontaneous postsynaptic activity and susceptibility to SLA generation all recordings in this part of study were made at room temperature (22–24°C). All quantitative measurements were taken 4–6 min after drug application. From 100–220 spontaneous events were collected from each neuron. Recordings of mIPSCs and mEPSCs were made using the same approach as for spontaneous postsynaptic currents but in the presence of 1 μ M tetrodotoxin (TTX) to block action potential-dependent synaptic release.

Patch-clamp recording in cell-attach configuration and field potential recordings were made using borosilicate glass capillaries filled with ACSF. All extracellular recordings were made at 32–34°C. Pipette resistance for these studies ranged from 1–3 M Ω . Field potential recordings were made from L2/3 and L5/6 somatosensory cortex using two-channel AC differential amplifier (A-M Systems, Carlsborg, WA) (bandpass 0.1 Hz–1 kHz; $\times$ 100). To induce SLA in the neocortex we used gabazine in concentrations of 1 μ M and 10 μ M. We did not find a significant difference in the effect of the two different concentrations of gabazine on probability of inducing SLA and frequency and amplitude of SLA recorded in slices from either control or flurothyl-treated rats. We therefore are presenting combined data obtained using 1 μ M and 10 μ M gabazine for both experimental groups, unless otherwise stated.

Data acquisition and analysis

The recordings were digitized (10 kHz) online with an analogue-to-digital converter Digidata 1322A (Axon Instruments). Data were analyzed using the Mini Analysis (version 5.5; Synaptosoft, Decatur, GA), Clampfit (Axon Instruments) and Origin 7.0 (Microcal Software, Northampton, MA, USA) software. The amplitude threshold for detection of sPSCs and mPSCs was set above the noise level and events were subsequently verified visually. The half-width and rise time of spontaneous and miniature postsynaptic currents were obtained by fitting from events without overlapping with other events. The amplitude and interevent interval of sPSCs and mPSCs were estimated for each event and then combined into two groups (control and flurothyl-treated) and averaged. Rise time and half-width of postsynaptic currents were estimated and averaged for each cell and the mean values were averaged and compared for control and flurothyl-treated rats.

Statistical differences were determined using the unpaired Student's *t*-test and Kolmogorov Smirnov (K-S) test. Data are expressed as the mean $\pm$ S.E.M; error bars also indicate S.E.M.

The number of cells was designated as “n”. Interictal-like activity was defined as high amplitude spikes in the EEG that occurred in isolation on a background of otherwise normal activity. Ictal-like activity consisted of rhythmic spikes with a frequency more than 1 Hz. SLA was defined as ictal or/and interictal activity. Proportions of slices with SLA were compared with the Fisher’s exact test.

Drugs

All drugs were delivered through the perfusate. D-APV, 6,7-Dinitroquinoxaline-2,3-dione (DNQX), TTX and gabazine (SR-95531) were obtained from Tocris (Ellisville, MO, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Results

Effect of neonatal seizures on probability to induce seizure in the neocortex

For determination of the long-term effects of neonatal seizures on neocortical function rats had 58–60 flurothyl seizures from P1 to P10 were then studied at P20–P30 and P60–P80 ($n = 10–12$; 2–4 slices per rat for each age) and compared with age-equivalent controls ($n = 9–15$; 2–3 slices per rat for each age). In field potential recordings from L2/3 and L5/6 of somatosensory cortex spontaneous SLA were not observed in either slices from controls or flurothyl-treated rats in either age group. It has been shown previously that neocortical seizures can be readily induced by chemical convulsants although much less efficiently than in hippocampus (Abdelmalik et al., 2005; Wells et al., 2000). We failed to induce SLA in neocortex in slices from flurothyl-treated rats at P60–80 using the 4-aminopyridine (6 slices) and low magnesium (5 slices) models of seizure. However, in field potential recordings from L2/3 of somatosensory cortex spontaneous interictal-like discharges were elicited when the tissue was perfused with gabazine, a specific blocker of GABAergic transmission, in ACSF. Ictal-like activity was generated in only 1 of 17 slices (slice from flurothyl-treated rat) (recording shown in Fig. 1A). As shown on Figure 1B, slices from rats with neonatal seizures were more prone to gabazine-induced seizures than slices from control rats at both ages. The probability of inducing SLA in neocortex decreased with age in both experimental groups. However the difference in probability of SLA between slices from control and flurothyl-treated rats increased with age (control vs flurothyl: $50.0\% \pm 7.5\%$ vs $76.5 \pm 7.3\%$ for P20–30 ($p = 0.037$, Fisher exact test), $14.7 \pm 6.2\%$ vs $38.1 \pm 7.5\%$ for P60–80 ($p = 0.005$, Fisher exact test)). Table 1 lists the different characteristics of SLA recorded in L2/3 somatosensory cortex from control and flurothyl-treated rats. The only characteristic which had significant difference was the increase in duration of oscillations SLA in slices from flurothyl-treated rats in response to $1\ \mu\text{M}$ gabazine. However when the concentration of gabazine was increased to $10\ \mu\text{M}$, the duration of SLA oscillations were increased to the same level for both experimental groups. Also the difference in duration of SLA induced by $1\ \mu\text{M}$ gabazine in slices from control and flurothyl-treated rats where not observed at P60–80.

Propagation of gabazine-induced seizures through layers and participation of different types of neurons in triggering and maintenance of SLA in L2/3

Simultaneous field potential recordings from L2/3 and L5/6 of somatosensory cortex were performed in slices from control and flurothyl-treated rats at P60–80. SLA was synchronously initiated by gabazine-contained ACSF in both layers in 12 of 17 slices (6 control and 6 flurothyl slices). By contrast, in 5 of 17 slices SLA were generated in L5/6 and propagated to L2/3 (2 control and 3 flurothyl slices) (Fig. 2). Even during the recording from the same slice the delay between L5/6 and L2/3 SLA varied considerably, with a range of $21.9 – 58.7\ \text{ms}$ and a mean value of $36.8 \pm 1.1\ \text{ms}$ for control and $35.8 \pm 1.6\ \text{ms}$ for flurothyl-treated rats. We did not have any preparations where SLA was generated in L2/3

and propagated to L5/6. We did not find a difference in a mean delay of oscillations between L2/3 and L5/6 in slices from control and flurothyl-treated rats ($p = 0.7$, Student's t test).

It has been shown previously that neocortical interneurons and pyramidal neurons could participate differently in generation and propagation of SLA induced by 4-aminopyridine (Rheims et al., 2008). Simultaneous cell-attached recordings from pyramidal cell or interneurons in L2/3 in combination with field recordings from L2/3 were performed on 5 control and 4 flurothyl-treated slices at P60–80. Recorded pyramidal cells showed action potentials during every event of SLA whereas interneurons were not involved in the initial step of SLA generation in all control as well as flurothyl treated group. Action potentials recorded from interneurons correlated with each SLA event beginning 1–2 min after SLA induction (Fig. 3). These results show that, unlike the maintenance phase where both neuronal subtypes were involved, the initiation phase of gabazine-induced seizure generation in L2/3 consisted of activity of pyramidal cells but not interneurons. This data is in agreement with a previous study using 4-aminopyridine model of ictogenesis in neocortex (Rheims et al., 2008). We did not find any differences in the contribution of pyramidal cells and interneurons to the initiation and maintenance of SLA in recordings from control and flurothyl-treated groups

Effect of neonatal seizures on spontaneous inhibitory and excitatory postsynaptic currents

We next examined whether flurothyl-induced neonatal seizures alter synaptic transmission in the neocortex. At P60–P80 sIPSCs and sEPSCs were recorded from visually identified neocortical pyramidal neurons in L2/3 of the somatosensory cortex using voltage-clamp techniques in a whole-cell configuration. sIPSCs were recorded as outward currents at 0 mV (reversal potential of EPSCs) and completely blocked by 10 μ M gabazine, indicating that they were mediated by GABA_A receptors.

Slices from rats which experienced recurrent flurothyl-induced neonatal seizures showed a clear reduction in the sIPSC amplitude compared with slices from untreated controls with a mean values 51.5 ± 0.7 pA ($n = 16$) for the control group and 47.7 ± 0.6 pA ($n = 18$) for the flurothyl-treated group ($p < 0.0001$, K-S test) (Fig. 4Ab). sIPSC interevent intervals in the flurothyl-treated group was 189.7 ± 6.5 ms ($n = 18$), which did not differ significantly from the sIPSC interevent interval in controls (190.3 ± 5.4 ms; $n = 16$, $p > 0.05$, K-S test) (Fig. 4Ac). The 10 – 90% rise time of sIPSCs was 2.9 ± 0.5 ms ($n = 16$) and 2.2 ± 0.4 ms ($n = 18$) in control and flurothyl group, correspondently. The half-widths of sIPSCs were also very similar in both groups (controls: 15.1 ± 1.9 ms, $n = 16$; flurothyl-treated: 16.4 ± 2.3 ms, $n = 18$).

The mean amplitude of sEPSCs recorded in cells from flurothyl-treated rats were significantly increased compared to controls (controls: 33.0 ± 0.6 pA, $n = 8$; flurothyl-treated: 39.9 ± 0.7 pA, $n = 9$ cells; $p < 0.0001$, K-S test) (Fig. 4Bb). The sEPSC interevent interval in the flurothyl-treated group were significantly decreased in the flurothyl-treated group compared to the controls (controls: 476.1 ± 14.0 ms, $n = 8$; flurothyl-treated: 368.3 ± 10.8 ms, $n = 9$; $p < 0.0001$, K-S test) (Fig 4Bc). Kinetic characteristics of sEPSCs recorded in cell from rats experiencing neonatal flurothyl seizures did not differ from sEPSCs recorded in cells from control rats in rise time (control: 1.5 ± 0.1 ms, $n = 8$; flurothyl-treated: 1.4 ± 0.1 ms, $n = 9$) or half-width (control: 5.5 ± 0.2 ms, $n = 8$; flurothyl-treated: 5.2 ± 0.6 ms, $n = 9$), indicating largely unchanged kinetic properties of sEPSC.

Adding the NMDA receptor antagonist D-APV (50 μ M) did not change significantly the sEPSC amplitude (31.8 ± 0.5 pA, $n = 13$, $p > 0.05$, K-S test), but significantly increased the interevent interval of sEPSC in the cells from control animals from 476.1 ± 14.0 ms ($n = 8$)

to 582.8 ± 13.4 ms ($n = 13$, $p < 0.0001$, K-S test) (Fig. 4Cb,c). In the flurothyl-treated group we found significant decreases in the amplitude of sEPSCs from 39.9 ± 0.7 pA to 31.5 ± 1.2 pA ($n = 15$, $p < 0.0001$, K-S test). The frequency of sEPSCs in the presence of D-APV significantly increased to 568.58 ± 16.9 ms ($n = 15$, $p < 0.0001$, K-S test) (Fig. 4Cc). Kinetic properties of sEPSCs recorded in the presence of D-APV remained unchanged in the control and flurothyl-treated group (data not shown).

Effect of neonatal seizures on miniature inhibitory and excitatory postsynaptic currents

Next we evaluated the effect of recurrent neonatal seizures on action potential-independent synaptic transmission. Recordings of mPSCs were made at the same age and used the same approach as for spontaneous postsynaptic currents but in the presence of TTX ($1 \mu\text{M}$), the voltage-gated sodium channel blocker. In contrast to sIPSCs we did not find any differences in the mIPSC amplitude in control (18.2 ± 0.1 pA, $n = 12$) and flurothyl-treated (18.5 ± 0.2 pA, $n = 14$) groups (Fig. 5Ab). However the mIPSC interevent interval in the flurothyl-treated group significantly increased compared to the controls (controls: 359.8 ± 7.8 ms, $n = 12$; flurothyl-treated: 417.5 ± 10.3 ms, $n = 14$; $p < 0.0001$, K-S test, Fig. 5Ac).

The amplitude of mEPSCs did not show difference in control and flurothyl-treated groups (Fig. 5Bb). As with the spontaneous EPSCs the interevent interval of mEPSCs recorded in cells from flurothyl-treated rats were significantly decreased compared to the controls (controls: 528.8 ± 11.5 ms, $n = 21$; flurothyl-treated: 435.7 ± 9.5 ms, $n = 14$; $p < 0.0001$, K-S test, Fig 5Bc).

Adding the NMDA receptor blocker D-APV in the same manner reduced the mEPSC amplitude in both the control and flurothyl groups (for controls: from 16.1 ± 0.2 pA, $n = 21$ to 15.3 ± 0.2 pA, $n = 10$, $p < 0.05$, K-S test; for flurothyl group: from 16.3 ± 0.2 pA, $n = 14$ to 15.2 ± 0.1 , $n = 11$, $p < 0.05$, K-S test, Fig. 5Ca). The interevent interval of mEPSCs recorded from cells in control group slightly increased after addition of D-APV in the extracellular solution (from 528.8 ± 11.5 pA, $n = 21$ to 544.4 ± 13.8 , $n = 10$, $p > 0.05$, K-S test). However, adding D-APV greatly increased the interevent interval of mEPSCs in flurothyl-treated group (from 435.7 ± 9.5 ms, $n = 14$ to 513.2 ± 12.4 ms, $n = 11$, $p < 0.0001$, K-S test). Under NMDA receptor blockade mEPSC interevent intervals in the flurothyl-treated group did not differ significantly from the mEPSC interevent interval in controls (Fig. 5Cc). We also did not find significant difference in the kinetic properties of mIPSCs and mEPSCs in the control and flurothyl-treated groups (mIPSC rise time and half-width for control: 3.1 ± 0.3 ms and 14.6 ± 0.4 ms, $n = 15$; and for flurothyl-treated group: 3.6 ± 0.5 ms, and 15.0 ± 0.6 ms, $n = 16$; and mEPSC rise time and half-width for control: 1.9 ± 0.1 ms and 6.8 ± 0.2 ms, $n = 21$; and for flurothyl-treated group: 1.7 ± 0.1 ms and 6.2 ± 0.4 ms, $n = 15$).

Discussion

In the present study by using electrophysiological techniques and the flurothyl model of epilepsy in rats we showed that recurrent seizures induced at an early age cause: i) long-term enhancement of susceptibility of neocortex to SLA generation that persists into adulthood; ii) decrease in amplitude of sIPSCs and increase of the interevent interval of mIPSCs in neocortical L2/3 pyramidal cells; iii) increase in frequency of sEPSCs and mEPSCs, and amplitude of sEPSCs; and d) an NMDA receptor-dependent effect of neonatal seizures on excitatory synaptic transmission in neocortical cells.

Whereas spontaneous seizures were not observed in any of the rats, more than 75% of slices from rats which experienced recurrent neonatal seizures developed neocortical SLA during perfusion with gabazine, which is 1.5 more frequent than can be induced in slices from age-

equivalent controls. Well et al. (2000) showed that when GABAergic neurotransmission was blocked in slices of neonatal mouse and rat neocortex, paroxysmal field potentials occurred in nearly half the slices from P4 to P7 neocortex. These results are consistent with our observations in slices from control rats at P20–30. The likelihood of inducing SLA with gabazine decreased with an age in both the controls and rats with a prior history of neonatal seizures. However, the difference in probability of inducing SLA in slices from flurothyl-treated and control rats became much more obvious with an age. We did not find difference in characteristics of induced SLA, propagation patterns of SLA through neocortical layers, or participation of different types of neurons in triggering or maintenance of SLA in L2/3 in slices from control and flurothyl-treated rats. These findings show that early life seizure do not influence the basic mechanism of generation and maintenance of SLA in local circuitry of somatosensory cortex.

The alteration of inhibitory synaptic transmission was in the same direction as in the neocortical neurons from young rats (Isaeva et al., 2009) and the CA3 pyramidal cells of the hippocampus (Isaeva et al., 2006). Short term and long-lasting downregulation of GABAergic transmission after increased neuronal activity has been reported in many laboratories (Gibbs, III et al., 1997a; Gibbs, III et al., 1997b; Blair et al., 2004; Goodkin et al., 2005). Several mechanisms have been proposed to explain this phenomenon: decreases in GABA_A $\alpha 1$ receptor subunit expression (Ni et al., 2004); increasing percentage of internalized GABA_A receptors (Goodkin et al., 2005); and receptor endocytosis which contribute to a decrease in receptor function (Blair et al., 2004). Also metabolic mechanisms such as partial dephosphorylation of the GABA_A receptor could be involved in decreases of GABA signaling (Whittington et al., 1995; Sanchez et al., 2005). As in our previous work on hippocampal neurons (Isaeva et al., 2006) we did not find significant differences in other measured parameters of sIPSCs suggesting that alterations of GABA signaling after flurothyl-evoked seizures in hippocampus and neocortex share the same mechanism. In the present study we did not find a difference in the amplitude of mIPSCs between control and flurothyl-treated rats, but found a significant difference in their interevent interval. This finding supports our previous data obtained from neocortical neurons in young rats (Isaeva et al., 2009), indicating that following flurothyl-induced neonatal seizures there is an alteration of presynaptic GABA transmission. Our data suggests that changes in GABAergic system occurring after neonatal seizures in somatosensory neocortex are permanent and the time course of the GABA signaling alteration during the aging of the animals was not affected.

In contrast to our previous hippocampal studies (Isaeva et al., 2006), sEPSC recorded from the neocortical pyramidal neurons showed significant differences in amplitude and frequency of spontaneous synaptic signals between the control and flurothyl-treated group. Adding the NMDA receptor blocker D-APV did not change the amplitude of sEPSCs in the control group. A recent study has shown that at the resting potential (~ -70 mV) NMDA current components comprise about 20 % of the charge transfer of an average mEPSC recorded in L4 pyramidal cell in the neocortex (Espinosa & Kavalali, 2009). Under our experimental conditions of a holding potential at -80 mV and 1,3 mM magnesium in the extracellular solution, the NMDA current component of an sEPSC should be expected to be near the baseline noise level, which explains the unaffected amplitude of sEPSCs by NMDA antagonists in the control group.

Unlike its effect on the amplitude of sEPSCs, application of D-APV significantly decreased the frequency of sEPSCs in neocortical cells from controls. A similar effect of this NMDA receptor antagonist was obtained for glutamate-mediated spontaneous excitatory postsynaptic currents in L2 neurons of the rat entorhinal cortex (Berretta & Jones, 1996). The authors connected this effect of D-APV with the presence of presynaptic NMDA

receptors which can tonically facilitate glutamate release in the CNS. Woodhall et al., (2001) have shown that these receptors are likely to be predominantly of the NR1 NR2B subtype and that they mediate frequency-dependent facilitation of glutamate transmission, probably by increasing intraterminal calcium via the receptor ionophore. Another explanation for the effect of D-APV on the sEPSC frequency is that blocking NMDA transmission in the network decreases the frequency of non-NMDA signaling due to reduced net activity (Leinekugel et al., 1997).

In our study following neonatal flurothyl seizures the amplitude and frequency of sEPSCs significantly increased comparatively to age-matched controls. This phenomenon could not be explained by an increase of glutamatergic postsynaptic receptors because mEPSC amplitude was not increased. The increase of the mEPSC frequency most likely reflects presynaptic alterations of glutamatergic transmission following neonatal seizures. Adding D-APV to the extracellular solution led to a decrease in the amplitude and frequency of sEPSCs and mEPSCs to the level observed in D-APV contained solution in the control group, suggesting an NMDA receptor contribution to this difference. These data are in agreement with observations made earlier by Yang et al., (2006) in the entorhinal cortex of chronically epileptic rats. They showed that the specific NR2B antagonist Ro 25-6981 decreased the frequency of sEPSCs in control rats to a lesser degree than in pilocarpine-treated rats. The authors stated that although function of presynaptic NMDA appears to be diminished in normal adult animals it is restored in epileptic adults and long-term increasing in frequency of sEPSCs in the entorhinal cortex of chronically epileptic rats most likely could be explained by an increase in presynaptic NMDA receptors. Interestingly, Yang and colleagues made their observations on rats with pilocarpine-induced chronic epilepsy. Our experiments have been performed on animals which experienced neonatal flurothyl seizures and do not developed electrographic seizures latter in life. Also, as opposed to the pilocarpine model of epilepsy, neonatal flurothyl seizures do not the result in cell loss (Rivielo et al., 2002).

In conclusion, our results show that recurrent flurothyl seizures induced in immature rats reduces the threshold to convulsant agent *in vitro*, indicating a persistent enhancement of neocortical excitability. The increased susceptibility to seizure generation in neocortical structure following neonatal seizures can be explained at least in part by an alteration in inhibition/excitation balance. Our results show that neonatal seizures similarly affect inhibitory synaptic system in both the neocortex and hippocampus. However, in contrast to our studies in the hippocampus, neonatal epileptic activity results in significant alterations in NMDA-dependent excitatory synaptic transmission in the neocortex. This alteration of both excitatory and inhibitory neurotransmission in the neocortex renders the neocortex quite susceptible to seizures. Our findings parallel the clinical situation whereas neonatal seizures frequently result in neocortical epilepsy.

Acknowledgments

Sponsored by NIH NINDS grant numbers: NS041595

Abbreviations

SLA	seizure-like activity
ACSF	artificial cerebrospinal fluid
AMPA	α -amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid
D-APV	D-2-amino-5-phosphonovalerate

GABA	gamma-aminobutyric acid
NMDA	N-methyl-D-aspartate
TTX	tetrodotoxin
DNQX	6,7-Dinitroquinoxaline-2,3-dione
sEPSCs	spontaneous excitatory synaptic currents
sIPSCs	spontaneous inhibitory synaptic currents

References

- Abdelmalik PA, Burnham WM, Carlen PL. Increased seizure susceptibility of the hippocampus compared with the neocortex of the immature mouse brain in vitro. *Epilepsia*. 2005; 46:356–66. [PubMed: 15730532]
- Acharya JN, Wyllie E, Luders HO, Kotagal P, Lancman M, Coelho M. Seizure symptomatology in infants with localization-related epilepsy. *Neurology*. 1997; 48:189–196. [PubMed: 9008517]
- Avanzini G, Franceschetti S. Cellular biology of epileptogenesis. *Lancet Neurol*. 2003; 2:33–42. [PubMed: 12849299]
- Berretta N, Jones RS. Tonic facilitation of glutamate release by presynaptic N-methyl-D-aspartate autoreceptors in the entorhinal cortex. *Neuroscience*. 1996; 75:339–44. [PubMed: 8931000]
- Blair RE, Sombati S, Lawrence DC, McCay BD, DeLorenzo RJ. Epileptogenesis causes acute and chronic increases in GABAA receptor endocytosis that contributes to the induction and maintenance of seizures in the hippocampal culture model of acquired epilepsy. *J Pharmacol Exp Ther*. 2004; 310:871–880. [PubMed: 15084648]
- Bo T, Jiang Y, Cao H, Wang J, Wu X. Long-term effects of seizures in neonatal rats on spatial learning ability and N-methyl-d-aspartate receptor expression in the brain. *Brain Res Dev Brain Res*. 2004; 152:137–142.
- Brunquell PJ, Glennon CM, DiMario FJ Jr, Lerer T, Eisenfeld L. Prediction of outcome based on clinical seizure type in newborn infants. *J Pediatr*. 2002; 140:707–12. [PubMed: 12072874]
- Chang YC, Huang AM, Kuo YM, Wang ST, Chang YY, Huang CC. Febrile seizures impair memory and cAMP response-element binding protein activation. *Ann Neurol*. 2003; 54:706–18. [PubMed: 14681880]
- Clancy RR, Legido A. The exact ictal and interictal duration of electroencephalographic neonatal seizures. *Epilepsia*. 1987 Sep-Oct; 28(5):537–41. [PubMed: 3653058]
- Clancy RR, Legido A, Lewis D. Occult neonatal seizures. *Epilepsia*. 1988 May-Jun; 29(3):256–61. [PubMed: 3371282]
- Cornejo BJ, Mesches MH, Coultrap S, Browning MD, Benke TA. A single episode of neonatal seizures permanently alters glutamatergic synapses. *Ann Neurol*. 2007; 61:411–426. [PubMed: 17323345]
- Dalby NO, Mody I. The process of epileptogenesis: a pathophysiological approach. *Curr Opin Neurol*. 2001; 14:187–92. [PubMed: 11262734]
- DeFazio RA, Hablitz JJ. Alterations in NMDA receptors in a rat model of cortical dysplasia. *J Neurophysiol*. 2000; 83:315–21. [PubMed: 10634874]
- Duchowny M, Harvey AS. Pediatric epilepsy syndromes: an update and critical review. *Epilepsia*. 1996; 37(Suppl 1):S26–S40. [PubMed: 8647050]
- Espinosa F, Kavalali ET. NMDA receptor activation by spontaneous glutamatergic neurotransmission. *J Neurophysiol*. 2009; 101:2290–2296. [PubMed: 19261712]
- Gibbs JW 3rd, Shumate MD, Coulter DA. Differential epilepsy-associated alterations in postsynaptic GABA(A) receptor function in dentate granule and CA1 neurons. *J Neurophysiol*. 1997; 77:1924–38. [PubMed: 9114245]

- Gibbs JW 3rd, Sombati S, DeLorenzo RJ, Coulter DA. Physiological and pharmacological alterations in postsynaptic GABA(A) receptor function in a hippocampal culture model of chronic spontaneous seizures. *J Neurophysiol.* 1997; 77:2139–52. [PubMed: 9114261]
- Glass HC, Glidden D, Jeremy RJ, Barkovich AJ, Ferriero DM, Miller SP. Clinical Neonatal Seizures are Independently Associated with Outcome in Infants at Risk for Hypoxic-Ischemic Brain Injury. *J Pediatr.* 2009 Sep; 155(3):318–23. [PubMed: 19540512]
- Goodkin HP, Yeh JL, Kapur J. Status epilepticus increases the intracellular accumulation of GABAA receptors. *J Neurosci.* 2005; 25:5511–5520. [PubMed: 15944379]
- Gulledge AT, Stuart GJ. Excitatory actions of GABA in the cortex. *Neuron.* 2003; 37:299–309. [PubMed: 12546824]
- Hauser E, Seidl R, Tenner W, Bernert G, Lischka A. Benign infantile familial convulsions. *Eur J Pediatr.* 1995; 154:499–500. [PubMed: 7671952]
- Holmes GL, Gairisa JL, Chevassus-Au-Louis N, Ben-Ari Y. Consequences of neonatal seizures in the rat: morphological and behavioral effects. *Ann Neurol.* 1998; 44:845–857. [PubMed: 9851428]
- Huang LT, Cilio MR, Silveira DC, McCabe BK, Sogawa Y, Stafstrom CE, Holmes GL. Long-term effects of neonatal seizures: a behavioral, electrophysiological, and histological study. *Develop Brain Res.* 1999; 118:99–107.
- Isaeva E, Isaev D, Khazipov R, Holmes GL. Selective impairment of GABAergic synaptic transmission in the flurothyl model of neonatal seizures. *Eur J Neurosci.* 2006; 23:1559–1566. [PubMed: 16553619]
- Isaeva E, Isaev D, Khazipov R, Holmes GL. Long-term suppression of GABAergic activity by neonatal seizures in rat somatosensory cortex. *Epilepsy Res.* 2009; 87:286–289. [PubMed: 19828295]
- Ishida S, Osawa T. Effects of postnatal electroshock convulsions on epileptogenesis of the amygdala and hippocampus in adult rats. *Acta Neurol Scand.* 1992; 85:128–131. [PubMed: 1574986]
- Karnam HB, Zhao Q, Shatskikh T, Holmes GL. Effect of Age on Cognitive Sequelae Following Early Life Seizures in Rats. *Epilepsy Res.* 2009a; 85:221–30. [PubMed: 19395239]
- Karnam HB, Zhou JL, Huang LT, Zhao Q, Shatskikh T, Holmes GL. Early Life Seizures Permanently Impairs the Hippocampal Map. *Exp Neurol.* 2009b; 217:378–87. [PubMed: 19345685]
- Legido A, Clancy RR, Berman PH. Neurologic outcome after electroencephalographically proven neonatal seizures. *Pediatrics.* 1991; 88:583–96. [PubMed: 1881741]
- Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R. Ca²⁺ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. *Neuron.* 1997; 18:243–55. [PubMed: 9052795]
- Martina M, Royer S, Pare D. Cell-type-specific GABA responses and chloride homeostasis in the cortex and amygdala. *J Neurophysiol.* 2001; 86:2887–2895. [PubMed: 11731545]
- Mathern GW, Pretorius JK, Mendoza D, Lozada A, Leite JP, Chimelli L, Fried I, Sakamoto AC, Assirati JA, Adelson PD. Increased hippocampal AMPA and NMDA receptor subunit immunoreactivity in temporal lobe epilepsy patients. *J Neuropathol Exp Neurol.* 1998; 57:615–34. [PubMed: 9630240]
- McBride MC, Laroia N, Guillet R. Electrographic seizures in neonates correlate with poor neurodevelopmental outcome. *Neurology.* 2000; 55:506–13. [PubMed: 10953181]
- McCabe PH, McNew CD, Michel NC. Effect of divalproex-lamotrigine combination therapy in frontal lobe seizures. *Arch Neurol.* 2001; 58:1264–8. [PubMed: 11493167]
- Mizrahi EM. Acute and chronic effects of seizures in the developing brain: lessons from clinical experience. *Epilepsia.* 1999; 40(Suppl 1):S42–S50. [PubMed: 10421560]
- Mizrahi EM, Clancy RR. Neonatal seizures: early-onset seizure syndromes and their consequences for development. *Ment Retard Dev Disabil Res Rev.* 2000; 6:229–241. [PubMed: 11107188]
- Morimoto K, Fahnestock M, Racine RJ. Kindling and status epilepticus models of epilepsy: rewiring the brain. *Prog Neurobiol.* 2004; 73:1–60. [PubMed: 15193778]
- Moshé, SL.; Velisek, L.; Holmes, GL. Developmental aspects of experimental generalized seizures induced by pentylenetetrazol, bicuculline and flurothyl. In: Malafosse, A.; Genton, P.; Hirsch, E.; Marescaux, C.; Broglin, D.; Bernasconi, R., editors. Idiopathic Generalized Epilepsies. John Libbey; London: 1994. p. 51–64.

- Neill JC, Liu Z, Sarkisian M, Tandon P, Yang Y, Stafstrom CE, Holmes GL. Recurrent seizures in immature rats: effect on auditory and visual discrimination. *Brain Res Dev Brain Res*. 1996; 95:283–92.
- Ni H, Jiang YW, Bo T, Wang JM, Pan H, Wu XR. Long-term effects of neonatal seizures on subsequent N-methyl-D-aspartate receptor-1 and gamma-aminobutyric acid receptor A-alpha1 receptor expression in hippocampus of the Wistar rat. *Neurosci Lett*. 2004; 368:254–257. [PubMed: 15364406]
- van den Pol AN, Obrietan K, Chen G. Excitatory actions of GABA after neuronal trauma. *J Neurosci*. 1996; 16:4283–4292. [PubMed: 8753889]
- Rheims S, Represa A, Ben-Ari Y, Zilberter Y. Layer-specific generation and propagation of seizures in slices of developing neocortex: role of excitatory GABAergic synapses. *J Neurophysiol*. 2008; 100:620–8. [PubMed: 18497363]
- Riviello P, de Rogalski LI, Holmes GL. Lack of cell loss following recurrent neonatal seizures. *Brain Res Dev Brain Res*. 2002; 135:101–104.
- Ronen GM, Buckley D, Penney S, Streiner DL. Long-term prognosis in children with neonatal seizures: a population-based study. *Neurology*. 2007; 69:1816–1822. [PubMed: 17984448]
- Sanchez RM, Koh S, Rio C, Wang C, Lamperti ED, Sharma D, Corfas G, Jensen FE. Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced seizures. *J Neurosci*. 2001; 21:8154–8163. [PubMed: 11588188]
- Sanchez RM, Dai W, Levada RE, Lippman JJ, Jensen FE. AMPA kainate receptor-mediated downregulation of GABAergic synaptic transmission by calcineurin after seizures in the developing rat brain. *J Neurosci*. 2005; 25:3442–3451. [PubMed: 15800199]
- Scher MS, Alvin J, Gaus L, Minnigh B, Painter MJ. Uncoupling of EEG-clinical neonatal seizures after antiepileptic drug use. *Pediatr Neural*. 2003; 28:277–280.
- Sogawa Y, Monokoshi M, Silveira DC, Cha BH, Cilio MR, McCabe BK, Liu X, Hu Y, Holmes GL. Timing of cognitive deficits following neonatal seizures: relationship to histological changes in the hippocampus. *Brain Res Dev Brain Res*. 2001; 131:73–83.
- Villeneuve N, Ben-Ari Y, Holmes GL, Gaiarsa JL. Neonatal seizures induced persistent changes in intrinsic properties of CA1 rat hippocampal cells. *Ann Neurol*. 2000; 47:729–738. [PubMed: 10852538]
- Wells JE, Porter JT, Agmon A. GABAergic inhibition suppresses paroxysmal network activity in the neonatal rodent hippocampus and neocortex. *J Neurosci*. 2000; 20:8822–8830. [PubMed: 11102490]
- Whittington MA, Traub RD, Jefferys JG. Erosion of inhibition contributes to the progression of low magnesium bursts in rat hippocampal slices. *J Physiol*. 1995; 486:723–734. [PubMed: 7473233]
- Woodhall G, Evans DI, Cunningham MO, Jones RS. NR2B-containing NMDA autoreceptors at synapses on entorhinal cortical neurons. *J Neurophysiol*. 2001; 86:1644–51. [PubMed: 11600627]
- Yamada J, Okabe A, Toyoda H, Kilb W, Luhmann HJ, Fukuda A. Cl⁻ uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. *J Physiol*. 2004; 557:829–841. [PubMed: 15090604]
- Yang J, Woodhall GL, Jones RS. Tonic facilitation of glutamate release by presynaptic NR2B-containing NMDA receptors is increased in the entorhinal cortex of chronically epileptic rats. *J Neurosci*. 2006; 26:406–10. [PubMed: 16407536]
- Zhao, Q.; Holmes, GL. Repetitive seizures in the immature brain. In: Pitkänen, A.; Schwartzkroin, PA.; Moshé, S., editors. *Models of Seizures and Epilepsy*. Elsevier; 2005. p. 341–350.
- Zhang G, Raol YS, Hsu FC, Brooks-Kayal AR. Long-term alterations in glutamate receptor and transporter expression following early-life seizures are associated with increased seizure susceptibility. *J Neurochem*. 2004; 88:91–101. [PubMed: 14675153]

Figure 1.

Different seizure susceptibility of somatosensory cortex in controls and rats with a prior history of neonatal seizures. Extracellular field potentials were recorded from L2/3 of somatosensory cortex in slices from P20–30 and P60–80 control and flurothyl-treated rats. A. Example of interictal (a) and ictal-like (b) activity evoked by 10 μ M gabazine in a slice from a flurothyl-treated rat at P71. B. Summary graphs show probability of SLA induction by gabazine in L2/3 of somatosensory cortex in slices from control (light grey) and flurothyl-treated (dark grey) rats at P20–30 (left) and P60–80 (right). Number of slices used for analysis shown in parenthesis. Note that in both age groups there was a significant increase in SLA probability in flurothyl-treated rats.

Figure 2.

Propagation of gabazine-induced seizures through layers of somatosensory cortex at P60–80. Examples of oscillations that occurred simultaneously in L2/3 and L5/6 (A) and oscillations initiated in L5/6 and propagated to L2/3 with a delay (B) in control group. Corresponding probability histograms of SLA delay in L2/3 vs L5/6 are shown below.

Figure 3. Participation of interneurons and pyramidal neurons in initiation and maintenance of gabazine-induced SLA in L2/3 of somatosensory cortex. Dual field potential recording and cell-attached recording from interneuron (A) or pyramidal cell (B) in slice from flurothyl-treated rat at P65. During the initial phase of gabazine-induced SLA pyramidal cell synchronously fired with field events while the interneurons remained silent. 1–2 min after the first oscillation occurs interneurons began to show action potential activity highly synchronized with field oscillations.

Figure 4.

Long-term effect of neonatal fluoroethyl-induced seizures on spontaneous inhibitory and excitatory synaptic transmission in L2/3 pyramidal cell of somatosensory cortex at P60–80. Aa. Example of sIPSCs recorded at holding potential 0 mV. Cumulative probability plots of amplitude (Ab) and interevent intervals (Ac) of IPSCs and corresponding bar graphs (mean $\pm$ SE) from control (light grey) and fluoroethyl-treated (dark grey) groups show decrease in amplitude of IPSCs in fluoroethyl-treated group, but no change in the interevent interval of sIPSC. Ba. Example of sEPSCs recorded at holding potential -80 mV in the presence of 10 μ M gabazine. Representative cumulative probability plots and bar graphs (mean $\pm$ SE) show increasing in frequency (Bc) and amplitude (Bb) of sEPSCs recorded in fluoroethyl-treated group (dark grey) vs control group (light grey). Number of cells used for analysis shown in parenthesis above each column. Ca. Example of sEPSCs recording at holding potential -80 mV in the presence of 10 μ M gabazine and 50 μ M D-APV. Representative cumulative probability plots and bar graphs (mean $\pm$ SE) show no difference in interevent interval (Cc) and amplitude (Cb) of sEPSCs in fluoroethyl-treated group (dark grey) vs control group (light grey) recorded with NMDA receptor blocker.

Figure 5.

Long-term effect of recurrent neonatal seizures on miniature inhibitory and excitatory synaptic transmission in L2/3 pyramidal cell of somatosensory cortex at P60–80. (Aa) Example of mIPSC recording (top and middle traces). Addition of gabazine to the extracellular solution completely blocks mIPSCs (bottom trace) (Ab and Ac) Cumulative probability of amplitude and interevent intervals and corresponding bar graphs of mIPSCs in flurothyl-treated group (dark grey) vs control group (light grey). (Ba) Recordings of mEPSC in control (ACSF) and during perfusion with D-APV and DNQX in a representative neuron. (Bb and Bc) Cumulative mEPSC amplitude and interevent distributions reveals a significant decrease in mEPSC interevent interval in flurothyl treated group. (Ca) Example of mEPSC recording in the presence of D-APV (top and middle traces) and D-APV and DNQX (bottom trace). Representative cumulative probability plots and bar graphs (mean $\pm$ SE) show no difference in interevent interval (Cc) and amplitude (Cb) of mEPSCs in flurothyl-treated group (dark grey) vs control group (light grey) recorded with NMDA receptor blocker. All values are means $\pm$ SEM.

Table 1

Effect of flurothyl-induced seizures on the characteristics of seizure-like activity induced by gabazine in L2/3 of the somatosensory cortex

Age and SLA Parameters	Control data	Slices (<i>n</i>)	Flurothyl data	Slices (<i>n</i>)
P20–30				
Frequency (Hz)*	0.08 ± 0.03	23	0.09 ± 0.02	27
Amplitude (mV)*	0.95 ± 0.07	23	1.02 ± 0.07	27
Duration (s) with 1μM gabazine	0.5 ± 0.05	10	1.15 ± 0.23 [†]	13
Duration (s) with 10μM gabazine	3.7 ± 0.75	13	3.03 ± 0.93	14
P60–80				
Frequency (Hz)*	0.07 ± 0.01	12	0.08 ± 0.02	32
Amplitude(mV)*	0.97 ± 0.2	12	0.77 ± 0.15	32
Duration (s) 1μM gabazine	5.25 ± 1.55	4	4.67 ± 0.35	14
Duration (s) 10μM gabazine	6.69 ± 1.11	8	7.25 ± 1.36	18

Data are presented as means ± SEM. P, postnatal day; SEM, standard error.

* The effects of flurothyl-induced seizures on the amplitude and frequency of SLA are presented as combinations of the effects induced by 1 and 10 μM gabazine. The effect on the duration of SLA was separately present for SLA induced by 1 and 10 μM gabazine.

[†] P < 0.05 vs. control, unpaired Student's t-test.