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# Selective impairment of GABAergic synaptic transmission in the flurothyl model of neonatal seizures

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## Abstract

Neonatal seizures can result in long-term adverse consequences including alteration of seizure susceptibility and impairment in spatial memory. However, little is known about the effects of neonatal seizures on developmental changes occurring in synaptic transmission during the first postnatal weeks. The purpose of the present study was to examine the effect of neonatal seizures on several aspects of γ-aminobutyric acid (GABA)ergic and glutamatergic synaptic transmission in the developing rat hippocampus. Flurothyl was used to induce multiple recurrent seizures in rat pups during the first postnatal days. Whole-cell patch-clamp recordings from the hippocampal CA3 pyramidal cell and extracellular recordings from the CA3 pyramidal cell layer were made in slice preparations. In rats that experienced neonatal seizures the amplitude of spontaneous inhibitory postsynaptic currents at P15–17 was decreased by 27% compared with controls, whereas neither frequency nor the kinetic properties were altered. Neonatal seizures did not affect the timing of the developmental switch in the $GABA_A$ signaling from excitatory to inhibitory. None of the studied parameters of glutamatergic postsynaptic currents was different between the flurothyl and control groups, including the amplitude and frequency of the spontaneous excitatory postsynaptic currents, the ratio of the amplitudes and frequencies of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA)-mediated spontaneous postsynaptic currents, and the kinetics of AMPA and NMDA mediated postsynaptic currents in the age groups P8–10 and P15–17. We suggest that the selective depression of the amplitude of GABAergic synaptic responses may contribute to the adverse neurological and behavioral consequences that occur following neonatal seizures.

## Introduction

While epileptic seizures can occur at any age, the newborn is at particularly high risk (Cowan, 2002). In addition to being common, neonatal seizures are associated with a significantly high risk for subsequent neurological sequela, including epilepsy, cognitive dysfunction and motor deficits (Bergman *et al*., 1983; Huttenlocher & Hapke, 1990; Ko & Holmes, 1999; Brunquell *et al*., 2002). While the cause of the seizures is an important determining factor for outcome, there is also evidence that seizures per se increase risk of brain injury, regardless of etiology (Rowe *et al*., 1985; Miller *et al*., 2002).

As in humans, rodent studies have also demonstrated that neonatal seizures result in substantial changes in the developing brain. Neonatal seizures are associated with an extensive synaptic reorganization of the axons and terminals of the dentate granule cells (Holmes *et al*., 1998, 1999; Huang *et al*., 1999), decreases in neurogenesis (McCabe *et al*., 2001), alterations in expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors (Sogawa *et al*., 2001),and impairment of visual spatial memory (Holmes *et al*., 1998; Huang *et al*., 1999; Chang *et al*., 2003) and auditory discrimination (Neill *et al*., 1996).

The detrimental effects of seizures may relate to their timing in regards to brain development. Neonatal seizures occur during periods of dramatic changes in neuronal connectivity and synaptic physiology. For example, the number of inhibitory and excitatory synapses increases (Fiala *et al*., 1998); γ-aminobutyric acid (GABA) action shifts from excitation to inhibition (Ben-Ari *et al*., 1989; Chen *et al*., 1996; Khalilov *et al*., 1999; Rivera *et al*., 1999; Lu & Trussell, 2001; Dzhala & Staley, 2003; Khazipov *et al*., 2004); the percentage of glutamatergic NMDA-receptor based 'silent' synapses decreases (Durand *et al*., 1996; Hsia *et al*., 1998); the decay of NMDA-mediated postsynaptic responses becomes faster (Carmignoto & Vicini, 1992; Hestrin, 1992; Khazipov *et al*., 1995); some membrane ionic channels and transporters appear and others disappear (Tarasenko *et al*., 1998; Rivera *et al*., 1999; Dzhala *et al*., 2005). Although the developmental changes in neuronal connectivity and synaptic physiology are largely guided by molecular mechanisms, internally generated spontaneous and experience-driven electrical activities in the developing brain exert a critical influence on synaptic maturation and refinement of neural circuits (Katz & Shatz, 1996; Ben-Ari *et al*., 1997; O'Donovan *et al*., 1998; Penn & Shatz, 1999). Alteration of the physiological patterns by paroxysmal activity may influence the developmental process. Indeed, there is increasing evidence that seizures can modify – slow down or accelerate – a wide range of unique processes that take place during development and are essential for the correct formation and wiring of the circuitry (Holmes *et al*., 2002).

In the present study we used the flurothyl model of epilepsy to investigate the influence of neonatal seizures on developmental changes

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in GABAergic and glutamatergic signaling in the CA3 pyramidal cells of the hippocampus. We evaluated the effects of neonatal seizures on: (i) developmental changes of spontaneous GABAergic and glutamatergic synaptic transmission; (ii) developmental switch of $GABA_A$ responses from excitatory to inhibitory; (iii) AMPA/NMDA spontaneous excitatory postsynaptic currents (sEPSCs) frequency and amplitude ratios; and (iv) influence of neonatal seizures on the kinetic properties of inhibitory and excitatory sPSCs.

## Materials and methods

### *Flurothyl epilepsy model*

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 were used throughout the experiments. The volatile agent flurothyl (2,2,2-trifluoroethyl ether; Aldrich Chemical, Milwaukee, WI, USA), a potent and rapidly acting CNS stimulant that produces seizures within minutes of exposure, was used to induce seizures.

The method used for flurothyl inhalation has been described previously in our laboratory (Huang *et al*., 1999; McCabe *et al*., 2001; Sogawa *et al*., 2001; Zhao & Holmes, 2005). Rats were placed in a plastic container (length, 28 cm, width, 18 cm, height, 26 cm), and liquid flurothyl was delivered through a plastic syringe and dripped slowly (3 cc/ h) onto filter paper in the center of the container where it evaporated. Rats were exposed to flurothyl until tonic extension of both the forelimbs and hindlimbs was observed. The end point was the tonic phase of the seizure rather than duration of time exposed to flurothyl as a variety of factors can influence time to seizure onset, such as respiratory rate, atmospheric pressure and ambient temperature. The pups were separated from their mother for about 10 min (5 min of flurothyl exposure and 5 min for recovery) every 2 h five times per day. The flurothyl-treated rats were returned to the mother when they appeared to be fully recovered from the seizure. Control rats were separated from the mother for equivalent amounts of time.

Two different protocols of flurothyl treatment were used for the patch-clamp recordings (studies of flurothyl effect on spontaneous postsynaptic transmission) and extracellular recordings (studies of flurothyl effect on developmental excitatory to inhibitory switch in the action of GABA). For the patch-clamp recordings rats were exposed to flurothyl five times per day for 5 days beginning at P1. From 3 to 11 days after the last seizure (age P8–P17) rats were killed to prepare slices for recordings (see below). To investigate alterations of the frequency, amplitudes and kinetic properties of spontaneous inhibitory postsynaptic currents (sIPSCs) and sEPSCs, we used seven to eight animals per studied group (P8–10 control and flurothyl, P15–17 control and flurothyl). We used from two to four slices per animal and one cell per slice. For extracellular recordings of multiple unit activity rats were exposed to flurothyl five times per day starting at P1 and continuing to P10 in a subset of animals. Rats exposed to flurothyl-induced seizures and age-match controls were studied from P5 to P21 onwards with a minimum of 24 h after the last induced seizure. We used 24 flurothyl-treated rats and 19 control rats for these experiments. Only one–two slices were evaluated from each rat.

To ensure that there was not a direct effect of flurothyl on postsynaptic currents, seven animals were exposed to subconvulsive doses of flurothyl (0.3 cc/ h). The amount of flurothyl in these studies was based on unpublished studies in which EEG monitoring was performed in the rat pups during flurothyl inhalation. Higher rates of infusion resulted in agitation, which was associated with EEG spikes. The infusion rate of 0.3 cc/h was used to assure that no epileptiform activity occurred. For this experimental group we used the same protocol of flurothyl treatment as for patch-clamp recordings and compared the effect of subconvulsive doses of flurothyl on amplitude of $GABA_A$ sIPSCs to control conditions at P15–17. We did not find any significant difference in this parameter between the two experimental groups of animals [$70.6 \pm 6.7$ pA at control ($n = 15$) vs. $74.8 \pm 8.1$ pA at subconvulsive doses of flurothyl ($n = 17$), $P > 0.05$].

### *Slice preparation*

On the day of the experiment both control and flurothyl-treated rats were deeply anesthetized using isoflurane and decapitated. The brains were removed and placed into an ice-cold solution of the following composition (in mM): sucrose, 250; KCl, 2; $CaCl_2$, 0.5; $MgCl_2$, 7; $NaHCO_3$, 26; $NaH_2PO_4$, 1.2; glucose, 11 (pH 7.4). Transverse hippocampal slices were cut using a Leica 1000S vibroslicer (Leica Microsystems, Nussloch GmbH, Germany). After dissection, slices were kept in an oxygenated (95% $O_2$–5% $CO_2$) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2.0; $MgCl_2$, 1.3; $NaHCO_3$, 25; $NaH_2PO_4$, 1.2; glucose, 11 (pH 7.3) at 30–32 °C for at least 1.5 h before use.

### *Patch-clamp recordings*

For recordings, slices were transferred to the recording chamber (Warner Instrument, Hamden, CT, USA) where they were fully submerged and superfused at 30–32 °C at a rate of 1–3 mL/min with the oxygenated extracellular solution, which contained (in mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 4.0; $MgCl_2$, 4.0; $NaHCO_3$, 25; $NaH_2PO_4$, 1.2; glucose, 11 (pH 7.3). A high divalent ion concentration (4/4 mM $Ca^{2+}/Mg^{2+}$) was used to block endogenous network activity (Groc *et al*., 2002).

sIPSCs and sEPSCs were recorded from visually identified hippocampal CA3 pyramidal cells using voltage-clamp technique in a whole-cell configuration. Patch electrodes were made from borosilicate glass capillaries (Garner Glass, Claremont. CA, USA) and filled with a solution of the following composition (in mM): Cs-gluconate, 117.5; CsCl, 17.5; NaCl, 8; HEPES, 10; EGTA, 10; $Na_3GTP$, 0.2; MgATP, 2 (pH 7.3). The recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Pipette resistances ranged from 5 to 7 MΩ, and seal resistances were 1–10 GΩ. The access resistances ranged from 10 to 18 MΩ and were compensated on 70–80% (lag 20 μs).

Spontaneous $GABA_A$ receptor-mediated PSCs were recorded at reversal potential of EPSCs (0 mV). In experiments studying the effects of flurothyl treatment on sEPSCs following successful whole-cell configuration every cell was held at 0 mV and incubated in ACSF containing 15 μM bicuculline until detectable spontaneous activity was blocked, and then recordings of sEPSCs were made at −80 mV and +40 mV to separately detect AMPA and NMDA sEPSCs on the same cell, respectively (Groc *et al*., 2002). It has been shown previously that at a holding potential of −80 mV the NMDA component of EPSCs is negligible in extracellular solution containing more then 2 mM $Mg^{2+}$ (Myme *et al*., 2003), so we did not use NMDA receptor blockers when measuring AMPA currents. At a holding potential of +40 mV the sEPSCs consist of both AMPA and NMDA components, so recordings were made in the presence of the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 10 μM in the extracellular solution. The remaining sEPSCs were completely abolished with the bath applica-

tion of the NMDA receptor antagonist D-(–)-2-amino-5-phosphonopentanoic acid (D-APV; 50 μM), indicating that sPSCs were mediated by NMDA receptors.

### *Extracellular recordings*

In a previous study (Khazipov *et al*., 2004) we described the method of estimation of the developmental switch of GABA action from excitatory to inhibitory. Briefly, extracellular recordings of the multiple unit activity (MUA) were obtained from CA3 pyramidal cell layer using metal electrodes of 50 μm diameter (California Fine Wire, Grover Beach, CA, USA) and the signal was amplified using a custom-made amplifier with enhanced electromagnetic interference noise suppression (bandpass 0.1 Hz−4 kHz; amplification 1000). Peak-to-peak noise was in the range of 20 μV. After 10–15 min of MUA registration the selective $GABA_A$ receptor agonist isoguvacine (Krogsgaard-Larsen & Johnston, 1978) was briefly applied on the slice (10 μM for 1 min, bath application) to activate $GABA_A$ receptors. In control experiments using whole-cell recordings from CA3 pyramidal cells we found that brief bath application of isoguvacine induced a transient chloride-dependent current sensitive to the $GABA_A$ receptor antagonist bicuculline. The effect of isoguvacine was associated with transient changes in the frequency of MUA and was completely reversible. We determined the effect of isoguvacine on MUA using a ratio of the MUA frequency at the peak of the isoguvacine response to the MUA frequency in control. For the purposes of fit we normalized the effect of isoguvacine by assigning values +1 and −1 for all values > 100% (excitation) and < 100% (inhibition), respectively.

### *Data acquisition and analysis*

For analysis of sPSC parameters, individual non-overlapping with other events PSCs were collected from recordings of at least 2–4 min duration at a given membrane potential. The average number of sPSCs collected per cell was 112.4 ± 12.2. The recordings were digitized (10 kHz) online with an analog-to-digital converter Digidata 1322A (Axon Instruments, Union City, CA, USA). Clampex and Clampfit (Axon Instruments), Mini Analysis (version 5.5; Synaptosoft, Decatur, GA, USA) and Origin 7.0 (Microcal Software, Northampton, MA, USA) software were used for the acquisition and data analysis. The threshold for sIPSC and sEPSC detection was set at twice the mean amplitude of the background noise. The frequency and peak amplitudes of sIPSCs and AMPA and NMDA sEPSCs were estimated and averaged for each cell, and the mean values were then averaged and compared for each age group in control and flurothyl-treated rats. The MUA was determined with a spike detection algorithm (Mini Analysis) and verified visually. Data are expressed as the mean ± tistical significance of difference between means was calculated using Student's *t*-test with a level of significance up to $P < 0.05$.

### *Solutions and drugs*

CNQX, D-APV, isoguvacine and bicuculline were obtained from Tocris (Ellisville, MO, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

## Results

### *Formation of functional GABAergic synapses*

In the first set of experiments we studied whether recurrent neonatal seizures alter the development of $GABA_A$-mediated synaptic transmission. We compared sIPSCs recorded from pyramidal cells of the CA3 region of the hippocampus in control and flurothyl-treated rats at P8–10 and P15–17. In the whole-cell patch-clamp recordings sIPSCs were observed as transient outward currents from a holding potential of 0 mV. Bath application of 15 μM bicuculline resulted in a complete reversible block of any detectable activity, indicating that sPSCs were mediated by $GABA_A$ receptors (data not shown). The frequency of sIPSCs in control rats increased significantly over the second and third postnatal weeks (Table 1) ($P < 0.005$). A similar increase in frequency of sIPSCs during this developmental period was also observed in flurothyl-treated rats ($P < 0.005$). As shown in Table 1, on average, the amplitude of sIPSCs in control rats also changed with advancing postnatal age ($P < 0.05$). In the flurothyl-treated rats, the amplitude of the sIPSCs did not differ from the control values at P8–10. However, sIPSCs amplitude did not increase during the third postnatal week and was 27% less than the amplitude of sIPSCs in age-matched control animals. We also examined whether neonatal seizures affect kinetic properties of sIPSCs by comparing rise time and half-width of average sIPSCs recorded from control and flurothyl-exposed pyramidal neurons. We found that these parameters were constant over the second and third postnatal weeks in control as well as flurothyl-treated rats (Table 1), with no significant difference between data obtained from these two groups of rats ($P > 0.05$).

### *Developmental excitatory-to-inhibitory switch in the $GABA_A$ signaling*

In the next series of experiments we asked whether recurrent neonatal flurothyl-induced seizures affect the developmental excitatory to inhibitory switch in the action of GABA. Whole-cell recordings are

TABLE 1. Effect of flurothyl-induced seizures on sPSCs parameters

| | Cells used (*n*)† | | Frequency (Hz) | | Amplitude (pA) | | Rise time (ms) | | Half-width (ms) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Age and sPSCs | Control | Flurothyl | Control | Flurothyl | Control | Flurothyl | Control | Flurothyl | Control | Flurothyl |
| P8–10 | | | | | | | | | | |
| $GABA_A$ | (17) | (21) | 3.1 ± 0.7 | 2.7 ± 0.4 | 49.8 ± 8.1 | 48.2 ± 7.4 | 0.8 ± 0.1 | 0.8 ± 0.1 | 9.3 ± 0.3 | 9.6 ± 0.7 |
| AMPA | (24) | (22) | 0.7 ± 0.1 | 0.6 ± 0.1 | 30.7 ± 2.4 | 35.8 ± 2.6 | 0.8 ± 0.1 | 0.9 ± 0.1 | 3.6 ± 0.1 | 3.5 ± 0.2 |
| NMDA | (24) | (22) | 0.4 ± 0.1 | 0.7 ± 0.1 | 30.4 ± 2.3 | 35.9 ± 2.3 | 10.2 ± 0.6 | 10.8 ± 0.5 | 56.5 ± 4.8 | 63.2 ± 5.3 |
| P15–17 | | | | | | | | | | |
| $GABA_A$ | (21) | (25) | 7.0 ± 0.6 | 6.5 ± 0.6 | 70.6 ± 6.7 | 51.8 ± 5.4*** | 0.7 ± 0.1 | 0.7 ± 0.1 | 10.2 ± 0.4 | 9.7 ± 0.4 |
| AMPA | (22) | (22) | 1.4 ± 0.2 | 1.8 ± 0.3 | 47.1 ± 3.6 | 50.9 ± 3.6 | 0.8 ± 0.1 | 0.8 ± 0.1 | 3.7 ± 0.2 | 3.8 ± 0.1 |
| NMDA | (22) | (22) | 1.1 ± 0.2 | 1.4 ± 0.3 | 50.1 ± 2.6 | 47.8 ± 3.8 | 4.3 ± 0.3 | 5.1 ± 0.0.5 | 22.9 ± 1.8 | 26.8 ± 2.9 |

†The numbers of cells used to measure frequency, amplitude and kinetic parameters of sPSCs for the control (cont.) and flurothyl (flur.) groups are presented in the first two columns in parentheses. Values are ± SEM. Statistical comparisons were between control and flurothyl group using Student's *t*-test, ***$P < 0.005$.

not suitable for studying the developmental switch in the action of GABA because the intracellular concentration of chloride ions is strongly affected by this type of recordings. We therefore used non-invasive extracellular recordings of MUA in the CA3 pyramidal cell layer. This method allowed us to record action potentials from tens of neurons in the vicinity of the recording electrode without altering the intracellular chloride concentration. To activate $GABA_A$ receptors, we briefly bath-applied the selective $GABA_A$ receptors agonist isoguvacine. In keeping with the results of a previous study (Khazipov *et al*., 2004), we found that isoguvacine caused a transient increase in MUA in slices from neonatal rats and a decrease in MUA in slices from older animals. Day-by-day analysis of the age dependence on the effect of the $GABA_A$ receptors activation of the MUA frequency is shown in Fig. 1A, in which the effect of isoguvacine is presented as a ratio between MUA frequencies at the maximum of the isoguvacine-induced response and during baseline control condition prior to isoguvacine application. Age dependence of the effect of isoguvacine on MUA was approximated with a Boltzmann function (Fig. 1B). A developmental switch in the action of isoguvacine from excitation to inhibition as determined by the midpoint of the Boltzmann fit (i.e. the age when in half of the slices isoguvacine induced an increase in MUA and in the other half a decrease in MUA) was estimated at P13.5 ± 0.4 in control rats ($n = 19$). In the rats that experienced flurothyl-induced seizures, the midpoint of the switch was at P14.1 ± 0.7 ($n = 24$). There was no statistical difference in the timing of the switch in the action of the $GABA_A$ agonist isoguvacine from excitatory to inhibitory between the control and flurothyl-treated groups ($P > 0.05$).

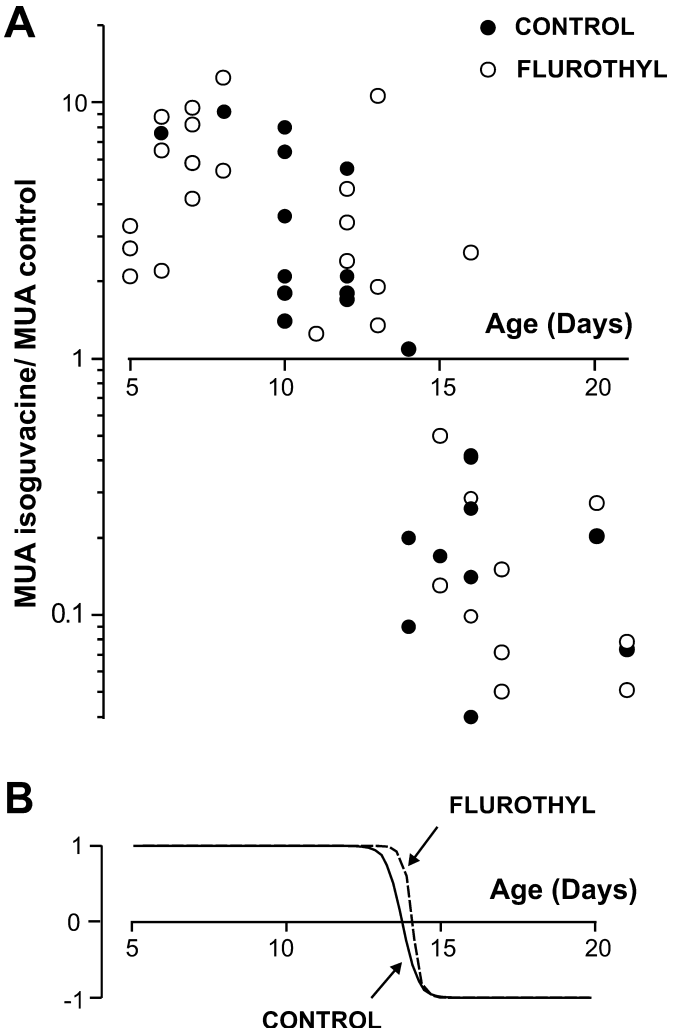


FIG. 1. Flurothyl-induced recurrent neonatal seizures do not affect the developmental switch in the action of GABA from excitatory to inhibitory. (A) Plot of the age dependence on the effect of the $GABA_A$ agonist isoguvacine on neuronal firing. Each point represents the ratio of the multiple unit activity (MUA) frequency at the peak of the isoguvacine effect to the MUA frequency in control. Pooled data from 22 and 30 slices prepared from control (filled circles) and flurothyl-treated (open circles) rats, respectively. Recordings were made from one–two slices per rat. (B) Boltzmann fit of the age dependence of the effect of isoguvacine on neuronal firing; note that the switch in $GABA_A$ signaling from excitation to inhibition is not significantly modified in the animals that experienced recurrent flurothyl-induced seizures.

### *Formation of functional glutamatergic synapses*

To investigate whether neonatal flurothyl-induced seizure influences excitatory synaptic transmission during the first weeks of postnatal development we recorded AMPA and NMDA sEPSCs. Recordings were made in the presence of 15 μM bicuculline and at holding potentials −80 and +40 mV to separately detect AMPA and NMDA sEPSCs, respectively. As shown in Table 1, the frequency of AMPA and NMDA sEPSCs recorded from control and flurothyl-treated rats increased significantly ($P < 0.05$) and to much the same extent over the second and third postnatal weeks for control rats and for flurothyl-treated rats. The average amplitudes of AMPA and NMDA sEPSCs also increased during this developmental period in control rats ($P < 0.05$). The amplitude of AMPA and NMDA sEPSCs recorded from flurothyl-treated rats increased with development to much the same extent as in control ($P < 0.05$). There were no significant differences between AMPA and NMDA sEPSCs amplitudes at any of the ages studied in the control and flurothyl-treated rats (Table 1).

### *AMPA and NMDA sEPSCs relationship*

Previous studies have suggested that during early postnatal development most glutamatergic synapses are pure NMDA receptor based and lack functional AMPA receptors (Isaac *et al*., 1995; Liao *et al*., 1995; Durand *et al*., 1996). The developmental conversion of silent to functional synapses is thought to be governed by activity-dependent (Liao *et al*., 1995; Durand *et al*., 1996; Liao & Malinow, 1996) and activity-independent mechanisms (Groc *et al*., 2003). We examined whether neonatal seizure can influence the process of activation of silent synapses by comparing, for each cell, the AMPA/NMDA sEPSCs frequency and amplitude ratios in slices from control and flurothyl-treated rats. Figure 2 shows that the AMPA/NMDA ratio for frequencies and amplitudes of sEPSCs recorded from CA3 pyramidal cells was constant over the second and third postnatal weeks in control and flurothyl-treated rats, with no significant difference between data obtained from these two groups of rats ($P > 0.05$).

### *Developmental changes in kinetic properties of sEPSCs*

Previous studies have indicated NMDA EPSCs become faster during early development (Carmignoto & Vicini, 1992; Hestrin, 1992; Monyer *et al*., 1994; Crair & Malenka, 1995; Khazipov *et al*., 1995; Shi *et al*., 1997) due to changes in the subunit composition of NMDA receptors (Monyer *et al*., 1992). It also has been shown that recurrent neonatal seizures may impair this process (Marianowski *et al*., 1995; Bo *et al*., 2004). To determine whether neonatal seizures induced by flurothyl influence the kinetic properties of sEPSCs we compared rise time and the half-width of AMPA and NMDA sEPSCs recorded in

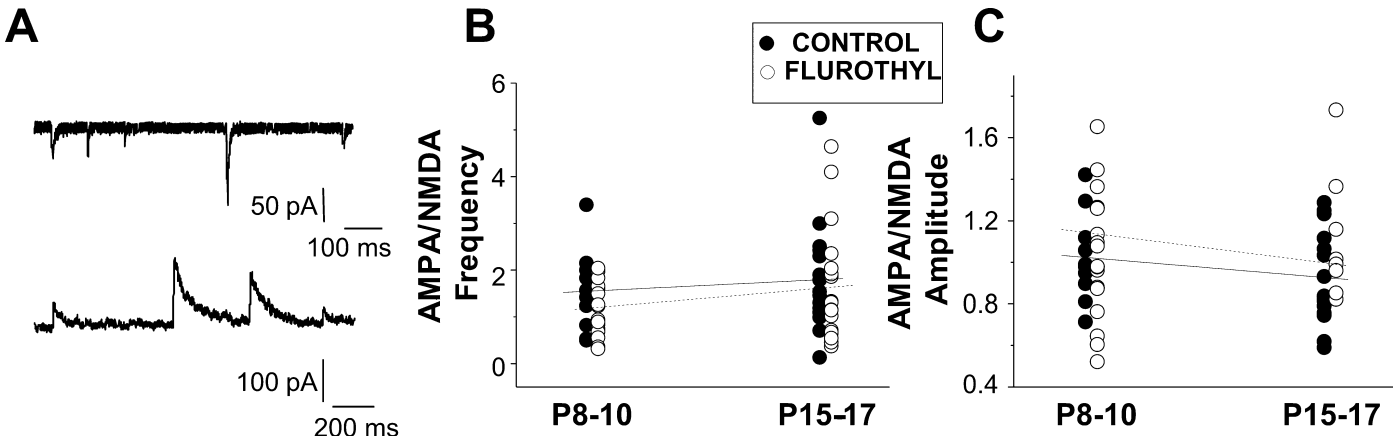


FIG. 2. Influence of neonatal seizures on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) sEPSCs relationship. (A) Spontaneous AMPA (upper panel) and NMDA (bottom panel) currents were recorded from the same cell at −80 mV and +40 mV, respectively. (B and C) The plots show that the AMPA/NMDA sEPSCs ratio for frequencies and amplitudes recorded from CA3 pyramidal cells was constant over the second and third postnatal weeks at control (filled circles, $n = 24$ at P8–10 and $n = 22$ at P15–17) and flurothyl-treated rats (open circles, $n = 22$ at P8–10 and $n = 22$ at P15–17). Each point represents the ratio of mean frequency (B) or amplitude (C) of AMPA vs. NMDA sEPSCs recorded from the same cell.

control and flurothyl-treated rats during the second and third weeks of postnatal development. As shown in Table 1, AMPA sEPSCs showed neither rise nor decay time changes with development in control as well as flurothyl-treated rats. NMDA sEPSCs in control rats significantly shortened with advancing postnatal age ($P < 0.05$ for rise time and $P < 0.005$ for half-width). There was no difference in developmental changes in rise time and half-width of NMDA sEPSCs recorded from CA3 pyramidal cells in control and flurothyl-treated rats during this period of postnatal development ($P > 0.05$; Table 1; Fig. 3).

## Discussion

We investigated the effect of recurrent neonatal seizure on developmental changes in inhibitory and excitatory synaptic transmission taking place in the early postnatal period using the flurothyl model of recurrent seizures *in vivo*. This period of brain development is associated with a rapid process of formation and maturation of neuronal network due to changes occurring in synaptic connectivity. In our study we found that both the frequency and amplitude of $GABA_A$-mediated sIPSCs as well as NMDA and AMPA sEPSCs in control rats greatly increases with advancing postnatal age. These phenomena directly correspond to an increased activity level of synaptic transmission (increasing number of contacts between neurons, amount of synapses per cell and maturation of synapses) (Pokorny & Yamamoto, 1981; Steward & Falk, 1991; Takumi *et al*., 1999; Lopez-Gallardo & Prada, 2001).

Recurrent neonatal seizures induced between P1 and P5 did not alter the trend of the developmental changes in the frequency of sIPSCs and sEPSCs for at least 2 weeks after the flurothyl-evoked seizures. Neonatal seizures also did not alter the developmental increase of excitatory synaptic current amplitudes for AMPA and NMDA sEPSCs. However, we found that the amplitude of sIPSCs was significantly diminished in rats undergoing recurrent neonatal seizures.

Downregulation of GABAergic synaptic transmission by flurothyl-induced seizures can be explained by different mechanisms. Recurrent seizures induced by flurothyl during the neonatal period result in a

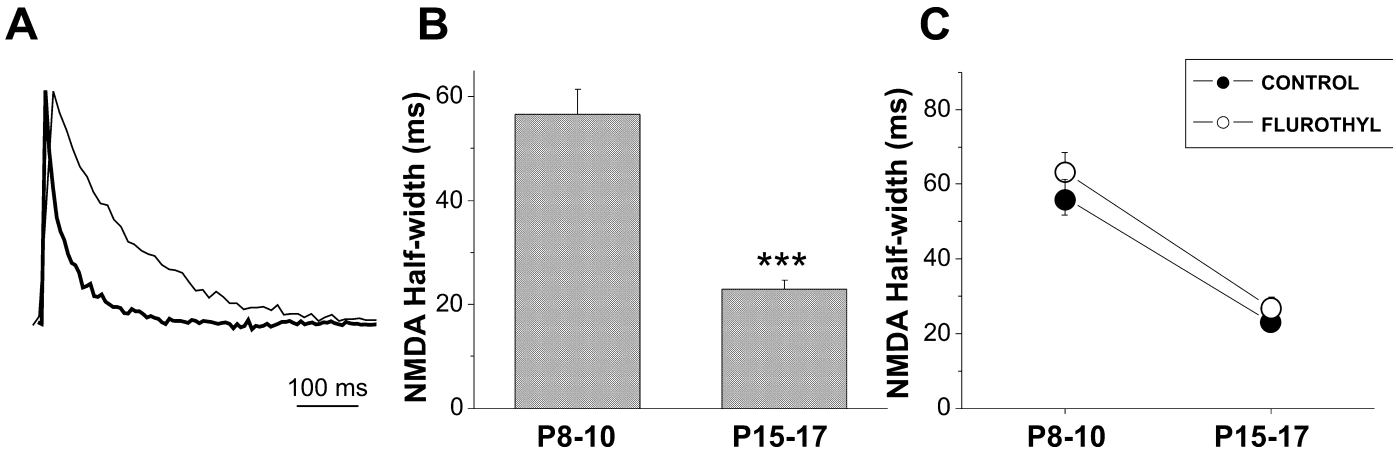


FIG. 3. Development changes of kinetics of *N*-methyl-D-aspartate (NMDA) sEPSCs inactivation recorded from CA3 pyramidal cells are not altered by flurothyl-induced seizures. (A) Normalized traces of averaged NMDA sEPSCs from control P8–10 (thin line) and P15–17 (thick line) hippocampi. (B) Summarized data from control hippocampi show that half-width of NMDA sEPSC was significantly shorter in P15–17 than in P8–10 pyramidal cells. (C) Alteration of NMDA sEPSC half-width during 2–3 postnatal weeks recorded from CA3 pyramidal cells from control (filled circles) and flurothyl-treated rats (open circles). Values are ± SEM. Statistical comparisons were performed using Student's *t*-test, ***$P < 0.005$.

long-term decrease in the expression of the $GABA_A$-α1 receptor subunit expression (Ni *et al*., 2004). Goodkin *et al*. (2005) showed that increased neuronal activity in hippocampal pyramidal culture increased the percentage of internalized $GABA_A$ receptors. These authors also showed that decreasing $MgCl_2$ or increasing KCl in the culture medium led to a 30% reduction in miniature (m)IPSC amplitude. It has been shown that $GABA_A$ receptor endocytosis can last at least several days after low-magnesium treatment of hippocampal culture and contribute to a decrease in receptor function and surface expression of $GABA_A$ receptors (Blair *et al*., 2004).

Another possible explanation for the downregulation of GABAergic synaptic transmission arises from studies demonstrating a postsynaptic decrease in $GABA_A$ receptor-mediated inhibition secondary to several different metabolic processes. In the low-magnesium model of epilepsy it was demonstrated that a postsynaptic decrease in $GABA_A$ receptor-mediated inhibition was ATP dependent (Whittington *et al*., 1995). It also has been shown that after hypoxia-induced seizures, CA1 pyramidal neurons exhibit a downregulation of $GABA_A$ receptor-mediated inhibition that was reversed by phosphatase calcineurin inhibitors (Sanchez *et al*., 2005).

Not all studies have demonstrated a decrease in inhibition following seizures in the neonatal period. Using patch-clamp recording and single-cell mRNA amplification techniques in single dentate granule cells of adult rats, Brooks-Kayal *et al*. (1998) demonstrated that the expression of $GABA_A$ receptor subunit mRNAs is substantially decreased in neurons from adult rats subjected to lithium-pilocarpine-induced status epilepticus. These changes in gene expression precede epilepsy onset by weeks and correlated with marked alterations in receptor function. However, using the same approach to induce status epilepticus in rats at P10 resulted in a twofold increase in $GABA_A$-α1 subunit expression (Zhang *et al*., 2004). It also has been shown that prolonged hyperthermia-induced seizures in the immature rat cause a selective presynaptic increase in inhibitory synaptic transmission in the hippocampus without alteration of excitatory synaptic transmission (Chen *et al*., 1999). The hyperthermia-induced seizures also permanently decrease seizures threshold and increase hippocampal excitability (Dube *et al*., 2000). The diversity of the experimental design used, including age of seizure induction, seizure duration and severity, and age killed makes it difficult to compare results. Our study tried to mimic the clinical situation of neonatal epilepsy by subjecting animals to multiple seizures per day, whereas most other studies examined the effect of a single long seizure.

Previous *in vitro* studies in the intact hippocampal preparation demonstrated that electroencephalographic seizures may result in a positive shift of the reversal potential of $GABA_A$-mediated currents and contribute to ictogenesis (Khalilov *et al*., 2003), similar to the chronic excitatory effect of GABA in a proportion of neurons in the subiculum in human temporal lobe tissue (Cohen *et al*., 2002). Although in the present study we did not measure the reversal potential of the GABA-mediated signals, our results indicate that the time course of the excitatory to inhibitory developmental shift in GABA signaling is not modified by recurrent flurothyl-induced neonatal seizures. The absence of change in the timing of the $GABA_A$ switch could be due to a lesser intensity of seizures in the flurothyl model compared with kainate-induced ictal discharges in the mirror focus model *in vitro*. In future studies it will be important to determine whether the developmental switch of GABA signaling from excitation to inhibition is modified in other models of recurrent neonatal seizures.

In the present study we also show that during the second and third weeks of postnatal development the kinetics of decay of NMDA sEPSCs significantly shortened. Similar to our findings, developmental changes in the kinetics of the NMDA receptor-mediated synaptic currents have been found in different regions of the brain (Carmignoto & Vicini, 1992; Hestrin, 1992; Laurie *et al*., 1992; Monyer *et al*., 1992; Khazipov *et al*., 1995; Barth & Malenka, 2001; Lu *et al*., 2001; Barria & Malinow, 2002; Townsend *et al*., 2003). Developmental changes in the properties of NMDA receptors are likely due to the switch in the molecular composition of NMDA receptors from NR2B to NR2A. In our study we did not found any significant difference in developmental changes in the NMDA sEPSCs decay kinetics recorded from control and flurothyl-treated rats. It has been shown that recurrent seizures induced by flurothyl in neonatal rats cause reduction of NR2A subunit expression and an increase of NR2C subunit expression in the cerebral cortex and hippocampus on P65 (Bo *et al*., 2004) These findings, combined with ours, suggest that seizure-induced changes in the ratios of NR2A and NR2C may occur over a long period of time following the seizures.

Another developmental feature of excitatory synaptic transmission that can be altered by neonatal seizures is the relative maturation of AMPA/NMDA signaling at newly formed glutamate synapses. It has been shown that a significant proportion of synapses in several regions of the immature brain display NMDA but not AMPA receptor activity. Conversion of such pure NMDA receptor and therefore presumably silent synapses to mixed NMDA and AMPA receptor synapses can occur via a long-term potentiation-like mechanism (Liao *et al*., 1995; Durand *et al*., 1996; Wu *et al*., 1996; Isaac *et al*., 1997). Our data show that during the second and third postnatal weeks the ratios of AMPA/NMDA sEPSCs were not altered in regard to either frequency or amplitude in control and flurothyl-treated rats. The role of neuronal activity on the maturation of AMPA/NMDA signaling has been a controversial subject, as experimental findings have supported opposite conclusions. Our data suggest that flurothyl-induced seizures induced in the critical period of brain development have no influence on maturation of glutamatergic synapses and support the hypothesis of activity independence of the development of AMPA/NMDA signaling (Friedman *et al*., 2000; Hohnke *et al*., 2000; Groc *et al*., 2003).

Our study adds further evidence that a seizure during early development subsequently alters synaptic function in the developing brain. We propose that the early pathophysiological changes could contribute significantly to the long consequences of seizures occurring early in life.

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## Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CA3, cornu ammonis region of the hippocampus 3; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-APV, D-(–)-2-amino-5-phosphonopentanoic acid; GABA, γ-aminobutyric acid; MUA, multiple unit activity; NMDA, *N*-methyl-D-aspartate; sEPSCs, spontaneous excitatory postsynaptic currents; sIPSCs, spontaneous inhibitory postsynaptic currents.

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