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Dissociated Gender-Specific Effects of Recurrent Seizures on GABA Signaling in CA1 Pyramidal Neurons: Role of $GABA_A$ Receptors

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Early in development, the depolarizing $GABA_A$ergic signaling is needed for normal neuronal differentiation. It is shown here that hyperpolarizing reversal potentials of $GABA_A$ergic postsynaptic currents (E_{GABA}) appear earlier in female than in male rat CA1 pyramidal neurons because of increased potassium chloride cotransporter 2 (KCC2) expression and decreased bumetanide-sensitive chloride transport in females. Three episodes of neonatal kainic acid-induced status epilepticus (3KA-SE), each elicited at postnatal days 4 (P4)–P6, reverse the direction of $GABA_A$ergic responses in both sexes. In males, 3KA-SE trigger a premature appearance of hyperpolarizing $GABA_A$ergic signaling at P9, instead of P14. This is driven by an increase in KCC2 expression and decrease in bumetanide-sensitive chloride cotransport. In 3KA-SE females, E_{GABA} transiently becomes depolarizing at P8–P13 because of increase in the activity of a bumetanide-sensitive NKCC1 (sodium potassium chloride cotransporter 1)-like chloride cotransporter. However, females regain their hyperpolarizing $GABA_A$ergic signaling at P14 and do not manifest spontaneous seizures in adulthood. In maternally separated stressed controls, a hyperpolarizing shift in E_{GABA} was observed in both sexes, associated with decreased bumetanide-sensitive chloride cotransport, whereas KCC2 immunoreactivity was increased in males only. $GABA_A$ receptor blockade at the time of 3KA-SE or maternal separation reversed their effects on E_{GABA}. These data suggest that the direction of $GABA_A$-receptor signaling may be a determining factor for the age and sex-specific effects of prolonged seizures in the hippocampus, because they relate to normal brain development and possibly epileptogenesis. These effects differ from the consequences of severe stress.

Key words: **seizure; patch clamp; $GABA_A$ receptor; development; hippocampus; histochemistry**

Introduction

Neonatal rats are more susceptible to seizures, but more resilient to seizure-induced acute injury or epileptogenesis, compared with adults (Moshé, 1993; Jensen, 1999; Galanopoulou et al., 2002; Holmes et al., 2002). Early life seizures or status epilepticus (SE) do not necessarily result in epilepsy (Moshé, 1993; de Rogalski Landrot et al., 2001; Holmes et al., 2002; Roch et al., 2002; Raol et al., 2006; Xiu-Yu et al., 2007). They may have, however, long-lasting consequences, potentially impacting on the level of functioning and behavior (Holmes, 1991; Sogawa et al., 2001; Hsu et al., 2003; Swann, 2004; Akers et al., 2006; Lai et al., 2006).

Among the signaling pathways with prominent role in brain development is the $GABA_A$ receptor system. In the embryonic and early postnatal brain, $GABA_A$ergic signaling is depolarizing and activates calcium signaling processes important for neuronal survival, migration, proliferation, and differentiation (Ben-Ari, 2002; Galanopoulou, 2005a). It gradually switches to its classical hyperpolarizing mode, after region- and cell type-specific tempos (Rivera et al., 1999). In sexually dimorphic structures like the substantia nigra, the timing of the switch is also gender specific (Galanopoulou et al., 2003a; Galanopoulou, 2005a, 2006; Kyrozis et al., 2006). The different developmental windows during which each neuronal structure is exposed to the neurotrophic and differentiating effects of depolarizing GABA further amplify the functional diversity across brain regions, ages, and sex. In the hippocampus of male rats or rats of unspecified sex, the timing of the functional switch of $GABA_A$ receptors has been placed around postnatal day 13.5 (P13.5) (Rivera et al., 1999; Khazipov et al., 2004; Banke and McBain, 2006). The direction of $GABA_A$ receptor signaling is determined by the activity of chloride cotransporters, which either decrease [like potassium chloride cotransporter 2 (KCC2)] or increase intracellular chloride [like sodium potassium chloride cotransporter 1 (NKCC1)] (Rivera et al., 1999; Farrant and Kaila, 2007). The gradual shift from an NKCC1-dominant state in immature neurons to a KCC2-dominant state in more mature neurons triggers this functional switch (Plotkin et al., 1997; Rivera et al., 1999; Farrant and Kaila,

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2007). In human epileptic temporal lobe tissue and adult rats subjected to seizures, an aberrant shift toward an NKCC1-dominant state and re-emergence of depolarizing $GABA_A$ergic signaling have been demonstrated and proposed to underlie certain epileptic discharges (Cohen et al., 2002; Rivera et al., 2002; Huberfeld et al., 2007).

To determine the effects of SE on the reversal potential of postsynaptic $GABA_A$ergic currents (E_{GABA}) in neonatal rat CA1 pyramidal neurons, the ontogeny and factors governing $GABA_A$ergic signaling were determined in naive neonatal male and female rats and in rats subjected to three episodes of neonatal kainic acid-induces SE (3KA-SE). The seizure effects were further differentiated from the effects of maternal separation. This report demonstrates sex-specific patterns of $GABA_A$ergic signaling in CA1 pyramidal neurons, identifies sex-specific effects of 3KA-SE on E_{GABA} and correlates them with the expression of KCC2 and NKCC1 chloride cotransporters, indicates that $GABA_A$ receptors mediate the 3KA-SE effects, and dissociates the effects of 3KA-SE and maternal separation.

Parts of this paper have been published previously in abstract form (Galanopoulou and Moshé, 2004; Galanopoulou, 2005b, 2007a,b,c).

Materials and Methods

Animals

The offspring of timed pregnant Sprague Dawley pups (Taconic Farms, Germantown, NY) that were used in this study were born in our animal housing facility and day of birth was considered as P0. Rats were maintained in our facility under standard procedures, in accordance with the Association for Assessment of Laboratory Animal Care guidelines. All procedures included in this study have been approved by the Animal Institute review committee. Rats were maintained in a 12 h alternating light/dark cycle and pregnant females were observed two to three times daily to monitor the time of delivery. Seizure induction and maternal separation were performed at P4–P6. Rats subjected to SE or maternal separation were kept at room temperature in cages together with five to six other pups, but without the dam, for 6 h daily with no access to food or water during P4–P6. They were subsequently returned to the dam. Selected female rats were weaned from the dam at P21, and each one was maintained in cages with one to two other female rats until they became 2–3 months old, with food *ad libitum*. After the electrode implantation for electroencephalographic (EEG) monitoring, rats were housed individually.

Experimental procedures

Gramicidin-perforated patch clamp. Male and female rats were deeply anesthetized with ketamine (100 mg/kg, i.p.), decapitated, and 330-μm-thick coronal sections through the anterior dorsal hippocampus were obtained (VT1000S vibratome; Leica, Nussloch, Germany). Procedures for harvesting and maintaining acute slices for electrophysiology have been described previously (Galanopoulou, 2006). To maintain chloride equilibrium, gramicidin-perforated patch clamp was used (Akaike, 1994; Kyrozis and Reichling, 1995; Galanopoulou, 2006; Kyrozis et al., 2006). Gramicidin-perforated patch clamp was performed at room temperature, in 95% O_2/5% CO_2 bubbled artificial CSF (ACSF) supplemented with the glutamate receptor inhibitors 6-cyano-7-nitroquinoxalene-2, 3-dione (CNQX; 10 μM) and 2-amino-5-phosphonopentanoic acid (AP5; 50 μM). Borosilicate glass electrodes were used, filled with electrode solution [containing (in mM) 77 K_2SO_4, 2 $MgCl_2 \times 6\ H_2O$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, 305 mOsm, pH 7.3] that contained the cation-permeable ionophore gramicidin. Initial experiments were done using 1–5 μg/ml gramicidin (Sigma, St Louis MO). However, because of change in the gramicidin lot, its concentration was subsequently adapted to 30 μg/ml, so as to maintain good access. Preparation of gramicidin stock and final working solutions for gramicidin were otherwise as described previously (Galanopoulou, 2006). A stainless-steel bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed within the stratum radiatum, along the visual centripetal pathway of pyramidal neuronal axonal processes, and at 100–200 μm approximate distance from the neurons of the CA1 pyramidal layer. To obtain postsynaptic currents (PSCs), electrical stimulation with 100 μs pulses was done on command. Neurons were approached under visual guidance, and were patched to obtain an initial seal >1 GΩ. Subsequently they were maintained under voltage clamp with a holding voltage of −70 mV, until access resistance improved. Slices were then incubated in ACSF with glutamate inhibitors (AP5, 50 μM and CNQX, 10 μM), to isolate the $GABA_A$ receptor-mediated PSCs. Analysis of the reversal potential for $GABA_A$ receptor-mediated PSCs (E_{GABA}) was done when access resistance was <100 MΩ (usually 35–55 MΩ) and E_{GABA} measurements were stable. To this end, 5–10 mV voltage steps were applied under voltage-clamp mode (range, −110 to −30 mV) around the estimated E_{GABA}, and neurons were stimulated at each voltage. Current–voltage (*I–V*) curves were then constructed by plotting the peak current after stimulation (15–25 ms after stimulation) versus the holding voltage at the same step; E_{GABA} was determined as the voltage at which no current was passing through. The average E_{GABA}, as estimated from three to five such attempts, was accepted, provided that the results were stable within 2 mV range. In addition, neurons were studied under current clamp recordings (holding current, 0 pA) to study firing rates and resting membrane potential (V_r). To determine whether the isolated PSCs were $GABA_A$ergic, at the end of the experiment, bicuculline 60 μM was bath applied. In the neurons included in this study, bicuculline abolished the observed PSCs. To indicate the direction of observed $GABA_A$ergic PSCs, measurements are presented as $E_{GABA} - V_r$. One and, rarely, two neurons per rat were studied. In the bumetanide experiments, a low dose of bumetanide (20 μM) was bath applied and $E_{GABA} - V_r$ was determined ~10 min later, when E_{GABA} had stabilized.

Induction of SE. Pups were weighed on P4, P5, and P6. Seizure induction was typically done between 2:00 P.M. and 8:00 P.M. to adjust for circadian cycle related differences in the responses. Seizures were induced with intraperitoneal injections of kainic acid (KA): 1.25 mg/kg at P4, 1.5 mg/kg at P5, and 2 mg/kg at P6 (KA456 groups). The incremental doses were necessary to ensure that rats would develop SE each day of seizure induction. Behavioral monitoring was done frequently to ensure that rats had continuous seizures: continuous until the onset of SE and every 30–60 min thereafter to monitor whether SE was maintained. Seizure progression included an initial stage with immobility followed by bouts of scratching behavior, hyperactivity and ataxia, focal limb myoclonus, and tonic or tonic-clonic seizures without interictal recovery. Behavioral seizures lasted for at least 5–6 h, starting to abate toward the end of the monitoring and separation period (6 h). Typically, most pups, especially on days P4 and P6, had seizures at the time of return to the cage. The morning after, the level of activity was similar to controls (CONs) and no behavioral seizures were observed at that time.

Maternal separation. To account for the potential effects of maternal separation, a different set of pups (SS456 groups) was injected with saline at the same time points and volumes as those used for the KA injections (P4–P6), and was kept separate from the dams for 6 h daily.

Effects of $GABA_A$ blockade. To test the role of $GABA_A$ receptors in the effects of 3KA-SE, separate groups of pups were injected with two bicu-

Table 1. Nomenclature of experimental groups used in this study

Group	Treatment
CON	Naïve controls, saline injected at P4, P5, and P6, but not separated from the dam
KA456	Rats subjected to KA-induced SE and undergone 6 h daily separation from the dam at P4, P5, and P6
SS456	Rats saline-injected at P4, P5, and P6, at the onset of their 6 h daily maternal separation period
BB	Rats injected with bicuculline 2 mg/kg (i.p.) 10 min prior to each KA injection and 6 h later (P4, P5, P6), but did not undergo maternal separation
BKB	Rats injected with bicuculline 2 mg/kg (i.p.) 10 min prior to each KA injection and 6 h later (P4, P5, P6); otherwise similar to KA456
BSB	Rats injected with bicuculline 2 mg/kg (i.p.) 10 min prior to each saline injection and 6 h later (P4, P5, P6); otherwise similar to SS456

Table 2. Ontogeny of E_{GABA} changes in male and female rat CA1 pyramidal neurons

Groups	*n*	E_{GABA} (mean ± SEM; mV)	*p*	$E_{GABA} - V_r$ (mean ± SEM; mV)	*p*
Male P4–P7	7	−58.7 ± 3		8.3 ± 2.8	
Male P8–P13	7	−62.4 ± 3.1		5.6 ± 3	
Male P14–P18	8	−74.2 ± 2.9	<0.05**	−3.3 ± 2.8	<0.05**
Female P4–P7	12	−68 ± 2.4	<0.05*	−3 ± 2.4	<0.05*
Female P8–P13	17	−71.3 ± 2	<0.05*	−2.5 ± 1.2	<0.05*
Female P14–P18	6	−81.2 ± 3.3	<0.05**	−10.6 ± 3.2	<0.05**

E_{GABA} and $E_{GABA} - V_r$ were determined in P4–P18 male and female CA1 pyramidal neurons from CON rats, using gramicidin-perforated patch clamp. Significant sex- and age-related differences were observed. *Significant difference versus same age male group; **significant difference versus either of the P4–P7 or P8–P13 same-sex groups.

culline doses (BKB groups; 2 mg/kg, i.p.; 10 min before and 6 h after each KA injection). For comparison, respective controls and maternally separated pups received two daily bicuculline doses, as described in Table 1. Administration of the selected bicuculline doses in maternally separated rats typically did not produce overt behavioral seizure activity and only rarely (8% of tested rats) infrequent and brief myoclonic or tonic seizure-like behaviors were observed, which did not evolve into SE. In contrast, in KA-injected rats, bicuculline (BKB groups) exacerbated the severity of KA-induced behavioral seizures, as rats manifested more intense clonic or tonic-clonic seizure activity.

Fluoro-Jade B staining and immunochemistry. Rats were deeply sedated (pentobarbital 100 mg/kg, i.p.), transcardially perfused with formalin (Sigma), brains were dissected, fixed overnight in formalin and preserved in 30% sucrose in PBS until sunk (4°C), fast frozen at −35°C, and kept at −80°C until further use (Galanopoulou, 2006). Rats intended for Fluoro-Jade B studies were killed at P7. Rats intended for immunochemical analysis were killed at P10. Fluoro-Jade B staining (Schmued et al., 1997) was done on 40-μm-thick coronal sections, cut on a Microm cryostat (Microm International, Walldorf, Germany). The following primary antibodies were used for the chloride cotransporters: rabbit polyclonal anti-KCC2 against amino acid sequence 932–1043 of rat KCC2 (Millipore, Billerica MA) (Williams et al., 1999) and rabbit polyclonal NKCC1 antibody against a 22 amino acid sequence at the C terminus of rat NKCC1 (Millipore) (Moore-Hoon and Turner, 1998); both were obtained from Millipore. The specificity of these antibodies has been reported in previous studies (Moore-Hoon et al., 1998; Williams et al., 1999; Aronica et al., 2007). In brief, immunochemistry was performed on free-floating 40 μm sagittal sections containing the anterior-dorsal hippocampi. Sections were incubated in 1% hydrogen peroxide in PBS (30 min, room temperature), and blocking solution for 1 h. Blocking solution consisted of PBS with 10% normal goat serum (NGS), 0.1% Triton X-100, and 0.1% bovine serum albumin (BSA) in KCC2 assays. In NKCC1 experiments, sections were blocked with PBS with 10% NGS, 0.3% Triton X-100, and 0.1% BSA. Incubation with primary antibodies was carried at 4°C in a shaker incubator for 3 d (1:250 dilution of anti-KCC2 antibody in PBS with 1.5% NGS, 0.1% Triton X-100, and 0.1% BSA; 1:500 dilution of anti-NKCC1 antibody in PBS with 1.5% NGS, 0.3% Triton X-100, and 0.1% BSA). Finally, sections were incubated with biotinylated anti-rabbit IgG (heavy and light chains) secondary antibodies made in goat (1:200; Vector Laboratories, Burlingame CA) and development of the colorigenic peroxidase-based reaction was performed as per manufacturer's instructions, using the Vectastain Elite and the NovaRed substrate kit (Vector Laboratories) (Galanopoulou, 2006).

Densitometry. To minimize bias in the intergroup comparisons caused by interassay variability, each assay included sections from one brain of each group (male and female CONs, SS456, and KA456). Microphotographs, magnified 400×, of optical fields corresponding to the CA1 pyramidal region were captured via a Nikon (Melville, NY) Ellipse 1000M microscope, transferred via a Eikonix (Burlington, MA) 1400 Series digital imaging camera to a G4 computer (Apple Computer, Cupertino, CA). Microphotographs were labeled with codes to allow unbiased and blind to investigator assessment during densitometry. Codes were revealed at the end of the study. Signal densitometry of KCC2- and NKCC1-immunoreactive pyramidal neurons was done with the Scion (Frederick, MD) Imager 1.62c (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) software, after conversion to gray-scale images. A maximum of four optical fields per CA1 section were selected per brain. Because CA1 pyramidal neurons are densely packed in the CA1 pyramidal layer, and because, at times, the borders of neuronal bodies were not clearly visible, as in weakly stained P10 male control KCC2-immunoreactive CA1 layers, regions of interests over the CA1 pyramidal layers were selected for densitometry. Background measurements were also obtained from the same optical field, derived from regions outside the CA1 layer that did not have any visible specific staining. The subtracted value of the "mean CA1 pyramidal layer density" minus "mean background density" of the same optical field was used in the final analysis. An average of three to four sections per rat were used. To minimize interassay variability, all values were referred to as the "percentage of mean KCC2-immunoreactivity (-ir) or NKCC1-ir in the P10 male control group of the same assay." The obtained values were normally distributed and with comparable variance.

Statistics. Statistical comparison of the electrophysiological data were done with ANOVA (one-way or multifactorial with Dunnett's *post hoc*) or Student's *t* test as indicated (Statview and JMP7 softwares; SAS Institute, Cary, NC). The immunochemical densitometric measurements were analyzed with repeated measures ANOVA with Tukey *post hoc*. Statistical significance was set at 0.05.

EEG monitoring at P7. To characterize the electrographic correlates of KA-induced seizures, five male and four female P6 rats were anesthetized with isoflurane and epidural EEG electrodes were placed at P6 at the right frontal and bilateral occipital regions, using the EEG headmounts of the EEG/EMG system (Pinnacle Technology, Lawrence, KS). P6 was chosen for the surgery, as epidural EEG headmounts were better tolerated at this age compared with younger pups. Pups were allowed to recover overnight and they were injected with kainic acid (2 mg/kg, i.p.) at P7. EEG and video monitoring were done for 30–45 min before KA injection (baseline), 6 h post-KA injection, and for 4 h daily at P8–P10. Pups were returned to the dam after each monitoring period. Acquisition and analysis of the EEG was performed using the Sirenia software (Pinnacle Technology). Electrographic seizures were defined as EEG patterns of high-amplitude rhythmic activity or repetitive spikes with evolution in frequency or amplitude, that were at least three times higher than the baseline activities and lasted for at least 3 s.

EEG monitoring in adulthood. To determine whether spontaneous seizures occurred in adulthood as a result of the 3KA-SE, seven KA456 and three CON female rats underwent stereotactic surgery for implantation of left hippocampal electrodes at 2–3 months of age. Before surgery, rats were anesthetized with a mixture of ketamine (70 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.). A wire electrode (E363/3; PlasticsOne, Roanoke, VA) was placed at the left dorsal hippocampus, at the following coordinates: −4.2 mm anteroposterior, 2.6 mm left, −3.6 mm depth. Bilateral cortical screws were implanted at 2 mm anteroposterior, 3 mm lateral. A reference screw electrode was placed at the right frontal bone and a ground electrode in the midline anterior to the occipital suture. The electrodes were fixed to the skull with dental acrylic cement. EEG and video monitoring was started at least 2–3 d after surgery. EEG was recorded 7 h a day (9:00 A.M. until 4:00 P.M.), 7–10 d per rat. Video monitoring was done for an average of 2–3 h daily per rat (Lado et al., 2001). EEG recording and analysis was done with the Stellate Harmonie software (Stellate Systems, Montreal, Quebec, Canada). At the end of the monitoring period, rats were killed with lethal doses of pentobarbital, and brains were collected and Nissl stained to confirm that the placement of the electrode was indeed in dorsal hippocampus.

Results

Ontogeny of E_{GABA} changes in male and female naive rats

To investigate the effects of 3KA-SE on E_{GABA}, the ontogenetic patterns of E_{GABA} changes were first determined in P4–P18 male and female CON pups. The following age groups were compared: P4–P7, P8–P13, and P14–P18. The V_r was similar between sexes

($F_{(1,55)} = 0.85$) and across ages ($F_{(2,54)} = 2.3$). In contrast, significant sex-specific developmental changes were noted in E_{GABA} and $E_{GABA} - V_r$, as presented in Table 2 and Figure 1.

E_{GABA} was significantly more negative in females than in age-matched males ($F_{(1,55)} = 11$, $p < 0.01$), and this difference was more pronounced in the P4–P14 groups (i.e., before the time of E_{GABA} switch in males) (Table 2, Fig. 1, supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The sex difference in E_{GABA} was observed for both P4–P7 and P8–P13 age groups, but not at P14–P18. In addition, in both sexes, E_{GABA} was significantly more negative in P14–P18 rats than in P4–P7 or P8–P13 rat CA1 pyramidal neurons of the same sex ($F_{(2,54)} = 11.2$, $p < 0.001$). No significant differences were noted in E_{GABA} between P4–P7 and P8–P13 same-sex rats.

Similar to E_{GABA}, sex and age differences were noted in $E_{GABA} - V_r$ (sex, $F_{(1,55)} = 16$, $p < 0.001$; age, $F_{(2,54)} = 6.6$; $p < 0.01$). In males, depolarizing $E_{GABA} - V_r$ values were observed before P14 in 13 of 14 neurons (Fig. 1). Using best-fit analysis, the age of E_{GABA} switch to hyperpolarizing values was estimated at P13.7. Between P14 and P18, six of eight studied male CA1 neurons had hyperpolarizing E_{GABA} values (Fig. 1). In both P4–P7 and P8–P13 age groups, females had typically negative or isoelectric $E_{GABA} - V_r$, except for five neurons that exhibited mildly depolarizing potentials. As shown in Figure 1*A*, these were scattered between P7–P13. $E_{GABA} - V_r$ retained hyperpolarizing values in females at least until P18. These show that the functional maturation of $GABA_A$ receptor signaling to hyperpolarizing in females occurs earlier than in male rat CA1 pyramidal neurons.

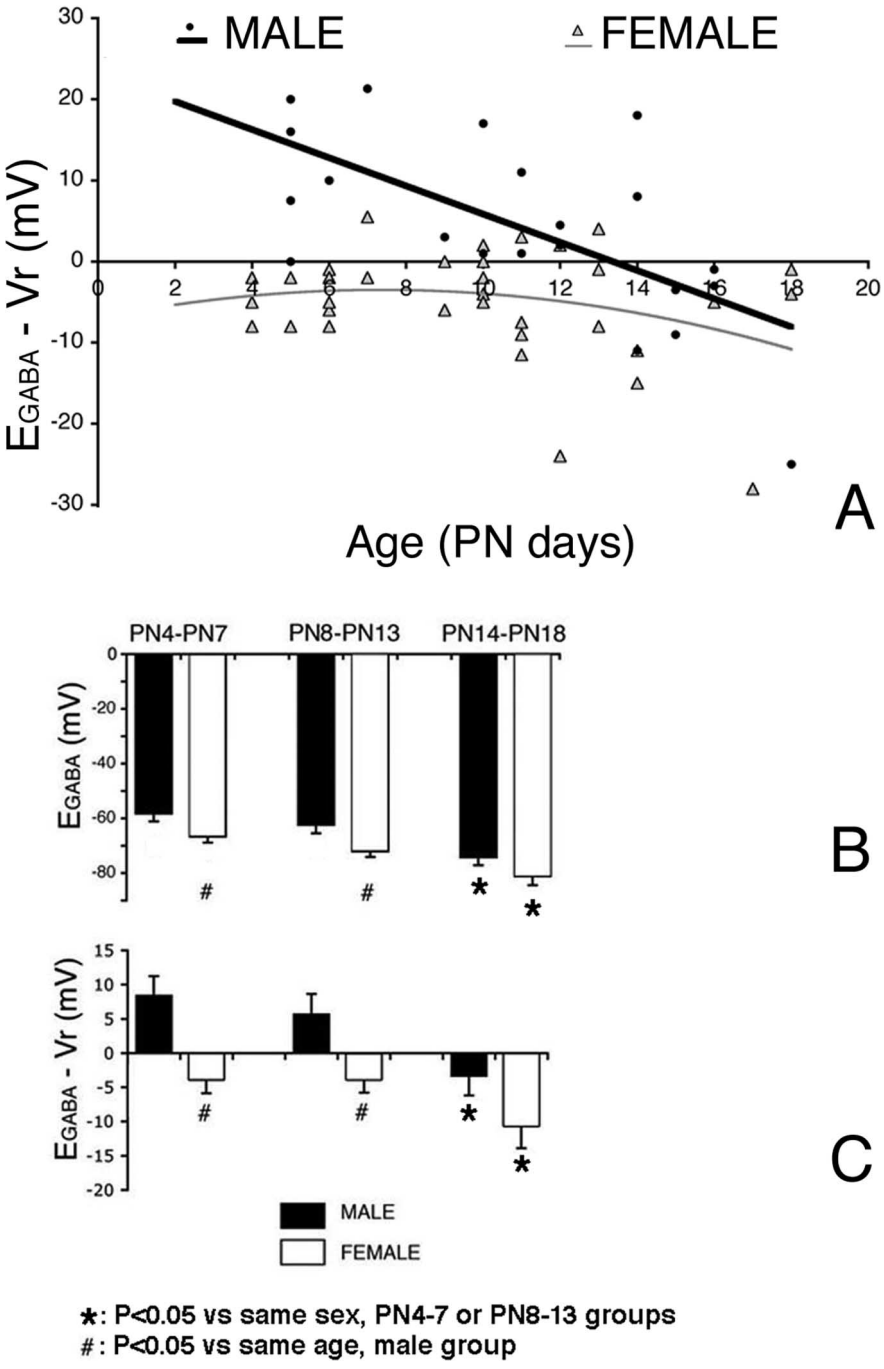

Figure 1. Sex differences in E_{GABA} in neonatal rat CA1 pyramidal neurons. ***A***, A scattergram of the $E_{GABA} - V_r$ values in control pups is depicted. Ninety-three percent of males (blue dots) had depolarizing $E_{GABA} - V_r$ values until P14 and subsequently switched to hyperpolarizing. Eighty-three percent of P4–P13 females (pink dots), however, already manifested hyperpolarizing or isoelectric responses by P4. ***B***, ***C***, Significant differences in mean E_{GABA} (***B***) and $E_{GABA} - V_r$ (***C***) between P4–P7, P8–P13, and P9–P14 male and female rat CA1 pyramidal neurons are noted. Results in ***B*** and ***C*** represent the mean ± SEM. PN, Postnatal day.

Characterization and effects of 3KA-SE

Characterization of KA-SE induced at P4–P6

Based on the above findings, it was decided to induce KA-SE at a time when the direction of $GABA_A$ receptor signaling was different in male and female CA1 pyramidal neurons, i.e., before P14. Because no significant E_{GABA} differences were found between P4–P7 and P8–P13 age groups, seizure induction at P4–P6 gave the additional advantage of allowing for an interim week, before the age when normal E_{GABA} switch occurs in males. Behavioral monitoring of pups injected with KA at P4 did not reveal any sex differences in the latency to onset of seizures. Latencies to onset of first tonic seizure were 24.8 ± 1.9 min in males ($n = 30$ rats) and 23.2 ± 2.2 min in females ($n = 29$ rats). Mortality was 5% in KA456 rats, occurring usually at P4–P7. No episodes of rejection by the dam were observed in the experimental groups, which also included SS456 pups. Growth curves were similar in CON, KA456, and SS456 groups (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). To determine whether neuronal injury occurred as a result of 3KA-SE, Fluoro-Jade B staining was done at P7 (Fig. 2). No significant presence of Fluoro-Jade B-stained cells in the CA1 pyramidal region or other hippocampal regions was noted in these groups ($n = 5$ male and female rats per group) (Fig. 2).

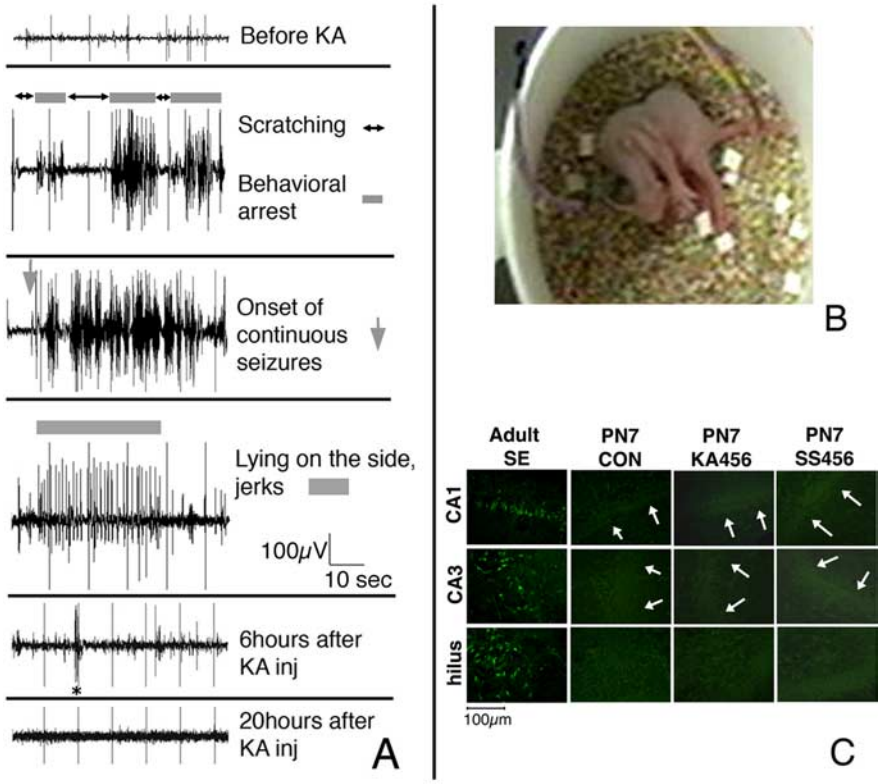

Figure 2. KA-induced seizures in neonatal rats evolve into SE but do not increase the number of Fluoro-Jade B-stained cells in the hippocampus. ***A***, ***B***, P7 male and female pups ($n = 9$) with epidural recordings were injected with kainic acid (2 mg/kg, i.p.) and monitored continuously for 6 h as well as for 4 h daily at P8–P10. The EEG derivations in ***A*** represent bipolar recordings from the right frontal and left occipital electrodes. Initially, bouts of scratching (without any change in EEG activity) interrupted by frequent periods of behavioral arrest that correlated with evolving high-amplitude EEG activity were noted. After the onset of continuous seizure activity (SE), tonic seizures (***B***) were also observed. Toward the end of SE, episodes of repetitive clonic jerks while the pup was lying on the side were also associated with EEG seizure patterns, as indicated by the gray bar. The ictal events became less frequent and interictal bursts of epileptiform activity were noted (asterisk). In eight of nine pups, no electrographic seizures were noted at P8–P10. ***C***, To determine whether the three episodes of KA-SE at P4–P6 resulted in neurodegeneration in the hippocampus, Fluoro-Jade B staining was performed. There were no Fluoro-Jade B-stained cells seen in the hippocampus. The figures are representative of $n = 5$ rats per group. As a positive control, the hippocampal sections from an adult rat subjected to a single episode of lithium-pilocarpine SE [methods described by Galanopoulou et al. (2003b)] 48 h before being killed are shown, which demonstrate many Fluoro-Jade B-stained (green) neurons in the hilus, CA1, and CA3 pyramidal layers. Magnifications are 400×. Scale bar, 100 μm. The white arrows indicate the borders of the CA1 and CA3 pyramidal regions. PN, Postnatal day.

EEG characterization of KA-SE

To characterize the electrographic correlates of the observed seizures, EEG monitoring using epidural electrodes was done in nine pups at P7 (five male and four female pups). Pups were injected with KA and monitored with video-EEG. Electrographic seizure activity appeared early in the course (10–15 min after KA injection) as frequent bursts of high-amplitude rhythmic activity (Fig. 2), coinciding with periods of immobility or behavioral arrest. These evolved into a continuous pattern of high-amplitude rhythmic activity intermixed with spikes or polyspikes that persisted for ~4.5–5.5 h. During this time, pups exhibited tonic or tonic-clonic seizures or episodes of ataxic movements and swimming-like behavior. Electroclinical seizures were still observed sporadically but less frequently by the end of the 6 h monitoring period. Follow-up EEG monitoring until P10 revealed electrographic seizures in only one female pup at P8 and P9. This pup had electroclinical seizures characterized by bursts of high-amplitude rhythmic activity associated with behavioral arrest followed by ataxic hindlimb alternating extensions at P8. Rare brief bursts of epileptiform activity, shorter than 10 s, were seen at P9 but not at P10, with no clear behavioral correlate.

Effects of KA-SE at P4–P6 on the direction of $GABA_A$ responses

To test whether early life 3KA-SE alter the direction of $GABA_A$ergic signaling, $E_{GABA} - V_r$ was determined in CA1 pyramidal neurons after P7 in pups subjected to 3KA-SE and their respective controls. Given the sex differences in the direction of the $GABA_A$ergic signaling in control neonatal CA1 pyramidal neurons, further assessment of the effects of neonatal seizures on $E_{GABA} - V_r$ was done separately for males and females. The effect of treatment (control, 3KA-SE, maternal separation) was significant in both male ($F_{(2,30)} = 9.6$, $p < 0.001$) and female pups ($F_{(2,39)} = 25.44$, $p < 0.0001$). Results are summarized in Table 3 and Figure 3.

KA456 male pups manifested hyperpolarizing $E_{GABA} - V_r$ at an earlier age (P9) compared with CONs (switch at P14) (Fig. 3*A*), resulting in significantly more negative $E_{GABA} - V_r$ values at the time of sex-specific $GABA_A$ergic responses (P9–P14) ($p < 0.05$) (Fig. 3*B*, Table 3) ($p < 0.05$). $GABA_A$ responses remained hyperpolarizing until at least P19.

In contrast, female KA456 pups showed a transient positive shift in $E_{GABA} - V_r$ toward depolarizing values from P8 until P13 (13 of 14 studied females; $p < 0.05$ vs CONs) (Fig. 3, Table 3). After P14, however, $E_{GABA} - V_r$ values returned to hyperpolarizing values in 8/10 neurons (-6.4 ± 3.1 mV; $n = 10$), which were similar to female CONs.

These data indicate that 3KA-SE at P4–P6 have sex-specific effects on the direction of $GABA_A$ergic postsynaptic responses of rat CA1 pyramidal neurons. In males, neonatal 3KA-SE accelerate the E_{GABA} switch, whereas in females, 3KA-SE trigger a transient reappearance of depolarizing $GABA_A$ergic signaling.

KCC2 expression in CA1 pyramidal neurons as a function of sex and 3KA-SE

To explain the sex-specific effects of 3KA-SE on E_{GABA}, KCC2-ir in CA1 pyramidal neurons from male and female KA456 P10 pups was studied. P10 was chosen as it falls within the period of sex-specific $GABA_A$ responses.

The expression of KCC2-ir was increased in female CON CA1 pyramidal neurons (155.7 ± 12% compared with male pups, semiquantitative method; $p < 0.05$; $n = 5$ rats per group), in agreement with the earlier appearance of hyperpolarizing $GABA_A$ergic signaling in female CON pups.

3KA-SE had sex-specific effects on KCC2-ir. In male KA456 pups, KCC2-ir was increased (183.6 ± 18.3% of CON values, semiquantitative method, $n = 5$ rats) (Fig. 4). In contrast, 3KA-SE had no significant effect on KCC2-ir in female rat CA1 pyramidal neurons ($n = 5$ rats per group) (Fig. 4). Consequently, the observed negative shift in E_{GABA} in male CA1 pyramidal neurons after 3KA-SE can be explained on the basis of increased

KCC2-ir expression. To further investigate the basis of E_{GABA} changes in female KA456 rats, the expression and activity of NKCC1 were studied.

Sex- and 3KA-SE-specific expression and activity of NKCC1 in CA1 pyramidal neurons

To determine the contribution of NKCC1 in E_{GABA}, NKCC1 protein expression was initially compared semiquantitatively with immunochemistry (Fig. 5*A*). Neither gender nor treatment-specific changes in NKCC1-ir were found in P10 male or female CA1 pyramidal neurons. However, the activation of NKCC1 also depends on post-translational modifications that are not detected by the used anti-NKCC1 antibody, including protein phosphorylation. To directly assess the relative contribution of NKCC1-like chloride cotransport activity among groups, the bumetanide inhibition assay was used. Low doses of bumetanide (20 μM) were bath applied, as these are known to selectively inhibit NKCC1-mediated chloride cotransport. The bumetanide-induced shift in E_{GABA} was determined ($[E_{GABA}]_{bumetanide} - E_{GABA}$) (Fig. 5*B*, supplemental Fig. 3, available at www.jneurosci.org as supplemental material). $[E_{GABA}]_{bumetanide}$ was measured at the point of maximal bumetanide effect (10 min of bumetanide bath application). These studies were performed in P9–P14 pups, during the developmental period after 3KA-SE induction, when the sex- and treatment-specific differences in E_{GABA} were still observed.

In CON groups, $[E_{GABA}]_{bumetanide} - E_{GABA}$ was -10.6 ± 1.4 mV in male pups ($n = 4$ neurons) and -7.5 ± 0.7 mV in females ($n = 8$ neurons; $p < 0.05$ vs male CONs) (Fig. 5*B*). The increased bumetanide-sensitive chloride cotransport activity in male CONs may therefore contribute, in conjunction with the lower KCC2 expression, to the more depolarizing $GABA_A$ergic responses observed in them.

3KA-SE had sex-specific effects. Male KA456 pups had reduced $[E_{GABA}]_{bumetanide} - E_{GABA}$ (-7.25 ± 0.25 mV, $n = 4$ neurons; $p < 0.05$ compared with male CONs). In female KA456, $[E_{GABA}]_{bumetanide} - E_{GABA}$ was increased compared with female CONs (-10.1 ± 0.3 mV; $n = 5$ neurons; $p < 0.05$).

These data indicate that 3KA-SE decrease bumetanide-sensitive NKCC1-like chloride cotransport in males but increase it in females.

Role of $GABA_A$ receptors on the sex-specific 3KA-SE-induced changes in $GABA_A$ receptor signaling

Because 3KA-SE have different effects on E_{GABA} in neurons with predominantly depolarizing (i.e., male) versus hyperpolarizing/isoelectric E_{GABA} values (i.e., female), the possibility that $GABA_A$ receptors mediate these sex-specific effects on E_{GABA} was examined. The effects of neonatal 3KA-SE were tested in male and female pups in which $GABA_A$ receptor signaling had been inhibited at the time of 3KA-SE. To achieve this, rats were injected with two bicuculline injections (2 mg/kg, i.p. before and 6 h after each KA injection; BKB groups) and E_{GABA} was determined during the period of divergent $GABA_A$ responses (i.e., P9–P14 in male, P8–P13 in female rats). The experimental groups, including respective controls, are listed in Table 1. Detailed descriptions of the preliminary studies leading to this protocol and of the intergroup comparisons are included in the supplemental text (available at www.jneurosci.org as supplemental material). None of these treatments resulted in significant presence of Fluoro-Jade B-stained degenerating cells in the hippocampus (data not shown).

Table 3. Effects of treatment on $E_{GABA} - V_r$ of rat CA1 pyramidal neurons

	Saline injection at P4–P6		Bicuculline administration at P4–P6 (two daily doses)		
Group	*n*	$E_{GABA} - V_r$ (mean ± SEM; mV)		*n*	$E_{GABA} - V_r$ (mean ± SEM; mV)
Male P9–P14					
CON	10	5.5 ± 2.7	BB	9	1.0 ± 1.4
KA456	10	−2.2 ± 1.0**	BKB	6	4.2 ± 1.8*
SS456	12	−7.7 ± 2.2**	BSB	9	4.2 ± 2.0*
Female P8–P13					
CON	16	−2.5 ± 1.2	BB	9	2.2 ± 1.8
KA456	14	7.2 ± 1.5**	BKB	5	−4.8 ± 2.6*
SS456	13	−8.6 ± 1.8**	BSB	5	4.8 ± 1.6*

E_{GABA} and $E_{GABA} - V_r$ were determined in P9–P14 male and P8–P13 female CA1 pyramidal neurons of the tested experimental groups, using gramicidin-perforated patch clamp. *Significant difference versus the respective saline-injected, same-sex group that had the same treatment otherwise (i.e. CON, KA456, or SS456); **significant difference versus CON same-sex group.

The effects of two daily doses of bicuculline at P4–P6 were significant in both male ($F_{(1,55)} = 7.2$; $p < 0.01$) and female ($F_{(1,61)} = 7.7$, $p < 0.05$) pups (Table 3, Fig. 6). Bicuculline treatment (BKB group) (Table 3, Fig. 6) reversed the effects of 3KA-SE on $E_{GABA} - V_r$ in both male and female pups, suggesting that in both sexes, $GABA_A$ receptors mediate the effects of 3KA-SE on E_{GABA}. Interestingly, in otherwise naive pups, blockade of $GABA_A$ receptors with bicuculline (BB groups) (Table 3 and Fig. 6) abolishes the sex differences in E_{GABA}, shifting $E_{GABA} - V_r$ toward more depolarizing potentials (i.e., more similar to males).

Effects of maternal separation

Neonatal maternal separation causes a negative shift in E_{GABA}

In both sexes, maternal separation resulted in hyperpolarized $E_{GABA} - V_r$ compared with same-sex CON pups (Fig. 3, Table 3). The effects of maternal separation were clearly separated from those of 3KA-SE in female but not in male pups (Fig. 3).

Effects of maternal separation on KCC2 and NKCC1

To identify the molecular correlates of the negative E_{GABA} shifts in SS456 pups, the expression of KCC2-ir, NKCC1-ir, and bumetanide-sensitive chloride cotransport activity were determined.

KCC2-ir was increased in male SS456 CA1 pyramidal neurons compared with CONs, but was less than in KA456 pups (139.14 ± 15% of CON values; semiquantitative scale; $n = 5$ rats; $p < 0.05$ vs male CON and vs KA456) (Fig. 4). No changes in KCC2-ir were observed in female SS456 pups.

NKCC1-ir in CA1 pyramidal neurons of male and female SS456 pups showed no significant differences from either CON or KA456 groups. In contrast, $[E_{GABA}]_{bumetanide} - E_{GABA}$ values were reduced compared with same-sex groups in both males (-5.9 ± 0.7 mV, $n = 4$ neurons; $p < 0.05$ compared with male CON; no significant difference compared with male KA456) and females (-5.1 ± 0.8 mV; $n = 5$ neurons; $p < 0.05$ compared with female CON or KA456 groups) (Fig. 5).

These data indicate that prolonged maternal separation augments the hyperpolarizing $GABA_A$ responses in both sexes, yet through different molecular pathways. In male SS456 pups, this is effected by increase in KCC2-ir and less active bumetanide-sensitive chloride cotransport. In female SS456 pups, this is attributed only to lower bumetanide-sensitive chloride cotransport.

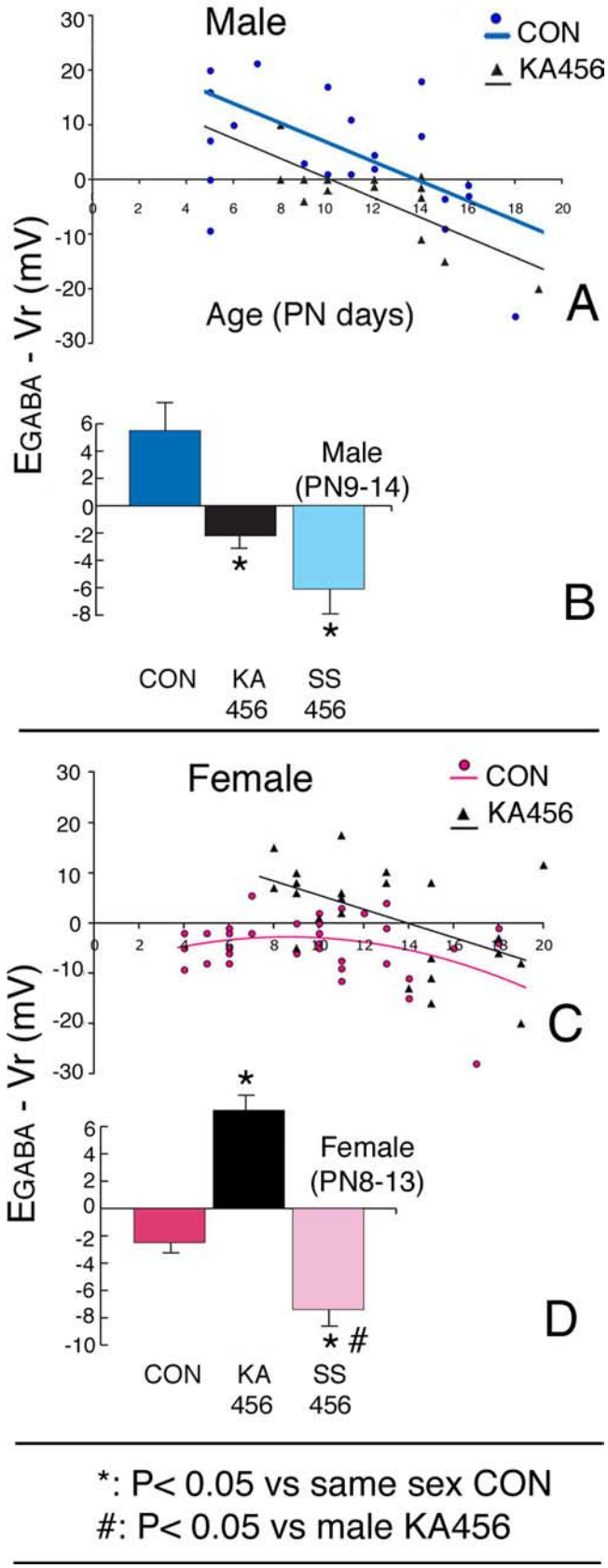

Figure 3. Effects of three episodes of neonatal KA-SE (KA456) and maternal separation on $E_{GABA} - V_r$ of male and female rat CA1 pyramidal neurons. ***A***, Male rats; scattergram of $E_{GABA} - V_r$ values according to age shows a negative shift in KA456 (black triangles) versus CON (blue circles) male rats. ***B***, Male rats; comparisons of $E_{GABA} - V_r$ among the three groups show that both KA456 and SS456 have hyperpolarizing $E_{GABA} - V_r$. ***C***, Female rats; scattergram of $E_{GABA} - V_r$ values according to age shows a transient reappearance of depolarizing $E_{GABA} - V_r$ in the KA456 group, between P8–13. ***D***, Female rats; comparisons of $E_{GABA} - V_r$ among the three groups show more depolarizing $E_{GABA} - V_r$ in KA456 pups and more hyperpolarizing $E_{GABA} - V_r$ in SS456 pups at P8–13. Results in ***B*** and ***D*** are shown as mean ± SEM. PN, Postnatal day.

Role of $GABA_A$ receptors in the effects of maternal separation on E_{GABA}

To determine whether the effects of maternal separation on E_{GABA} were also mediated through $GABA_A$ receptors, the effects of two daily doses of bicuculline (BSB groups) (Table 1) were studied. Bicuculline treatment reversed the effects of maternal separation in both sexes (BSB groups) (Table 3, Fig. 6). These indicate that, similar to 3KA-SE, $GABA_A$ receptor inhibition at the time of maternal separation can reverse its effects on E_{GABA} in both sexes, yet females are more sensitive, as they require only a single daily dose of bicuculline.

The transient aberrant switch of $GABA_A$ receptor signaling in female CA1 pyramidal neurons is not sufficient to trigger epileptogenesis

EEG and video monitoring was done in seven KA456 and three CON adult female rats to detect spontaneous seizures. No electrographic or behavioral seizures were observed, suggesting that the transient 3KA-SE induced reappearance of depolarizing $GABA_A$ signaling does not suffice to trigger epilepsy in adulthood.

Discussion

This is the first direct electrophysiological evidence in acute slices that the direction of $GABA_A$ receptor signaling in rat CA1 pyramidal neurons is sex specific in normal development and as a result of neonatal KA-SE. Maternal separation also influences E_{GABA}. Specifically, hyperpolarizing $GABA_A$ responses occur earlier in female neonatal rat CA1 pyramidal neurons than in male. Neonatal 3KA-SE reverse the direction of $GABA_A$ responses, via $GABA_A$ receptor-mediated pathways. E_{GABA} becomes hyperpolarizing in male and transiently depolarizing in female pups, after 3KA-SE, because of sex-specific changes in the expression or activity of chloride cotransporters. Maternal separation produces a negative shift in E_{GABA} in both sexes, but with sex-specific regulatory effects on chloride cotransporters.

The estimated age of E_{GABA} switch in male rats (P13.7) agrees with published studies using male or rats of undetermined sex (Khazipov et al., 2004; Banke and McBain, 2006). In most female CA1 pyramidal neurons, the switch has already occurred before P4. The rare depolarizing $GABA_A$ responses in females at P7–P13 may be random or attributed to the increase in carbonic anhydrase VII that favors depolarizing $GABA_A$ signaling (Rivera et al., 2005). These sex differences may explain the significant variability in $E_{GABA} - V_r$ measurements in previous studies, before E_{GABA} switch (Khazipov et al., 2004; Banke and McBain, 2006). As is the case in the substantia nigra (Galanopoulou et al., 2003a), the earlier switch is driven by the higher KCC2 expression in females. Additionally, there is less active bumetanide-sensitive chloride transport in female neurons. $GABA_A$ receptor-mediated neuronal depolarizations can activate L-type voltage sensitive calcium channels (Reichling et al., 1994; Owens et al., 1996; Galanopoulou et al., 2003a), initiating a cascade of calcium-sensitive signaling processes important for differentiation, plasticity, migration, proliferation, and neuronal communication (Ben-Ari, 2002). Therefore, in P4–P14 rat CA1 pyramidal neurons, $GABA_A$ receptor activation, under physiologic conditions, can promote

calcium-sensitive differentiation only in males, amplifying the already pre-existing sexually dimorphic phenotype of the neonatal hippocampus, as has been proposed for the substantia nigra neurons (Galanopoulou et al., 2003a; Galanopoulou, 2005a). Such events may contribute to the sexual dimorphism of the hippocampus, because it relates to morphology, connectivity, protein expression, and function (Cahill, 2006). Previously, sex-specific *in vivo* effects of high doses of the $GABA_A$ergic agonist muscimol on CREB (cAMP response element-binding protein) phosphorylation were reported in CA1 neurons (Auger et al., 2001). After the current manuscript was initially submitted, muscimol was reported to increase calcium in male but not in female neurons from mixed populations of hippocampal cells after 7 d in culture (Nunez and McCarthy, 2007).

3KA-SE at P4–P6 reverses the direction of $GABA_A$ signaling in both sexes by altering the expression of KCC2 and NKCC1. In males, 3KA-SE accelerated the E_{GABA} switch to hyperpolarizing values, whereas in females it caused a transient reappearance of depolarizing $GABA_A$ responses. This is not caused by deafferentation (Nabekura et al., 2002), because no neuronal injury was noted in P7 hippocampi, regardless of treatment. The female data are in accordance with studies on adult or immature neurons with hyperpolarizing $GABA_A$ergic signaling at the time of seizures; under these circumstances, seizures decrease the relative expression of KCCs over NKCC1, favoring a switch to depolarizing $GABA_A$ergic signaling (Rivera et al., 2002, 2004; Khalilov et al., 2003; Okabe et al., 2003; Wake et al., 2007). A determining factor for the effects of seizures on E_{GABA} is the direction of $GABA_A$ signaling at the time of SE. This is supported by the observation that bicuculline reverses the effects of 3KA-SE on E_{GABA} in both sexes. It has been reported that multiple daily, brief flurothyl-induced seizures in neonatal rats do not change the timing of $GABA_A$ receptor switch (Isaeva et al., 2006). This may indicate that prolonged but not brief seizures influence E_{GABA} switch. However, in that study, sex was not specified, and this may contribute to the lack of seizure effect on E_{GABA}. Indeed, if sex is not accounted for in the $E_{GABA} - V_r$ scattergrams presented herein (Fig. 3), E_{GABA} switch will occur around P14 in both CON and KA456 pups. Finally, the difference may be model specific. Flurothyl has $GABA_A$ receptor antagonistic effects (Krasowski, 2000). Consequently, flurothyl-induced seizures may be similar to our BKB groups, which counteract the effects of 3KA-SE on E_{GABA}.

At the molecular level, the 3KA-SE-induced appearance of precocious hyperpolarizing $GABA_A$ responses in males is caused by an increase in KCC2-ir and decrease in bumetanide-sensitive chloride transport. Indeed, activation of $GABA_A$ receptors during SE can depolarize CA1 neurons and upregulate KCC2 (Galanopoulou et al., 2003a). The transient depolarizing $GABA_A$ responses in females, after 3KA-SE, are secondary to increased activity of bumetanide-sensitive cotransporters. Alternatively, other factors that relate to seizures, like the brain-derived neurotrophic factor (BDNF) may have cell type-specific regulatory effects on KCC2 expression (Rivera et al., 2004). Because BDNF is released during seizures (Rivera et al., 2002), it would be interesting to test whether sex differences in BDNF signaling exist in P4–P6 CA1 pyramidal neurons.

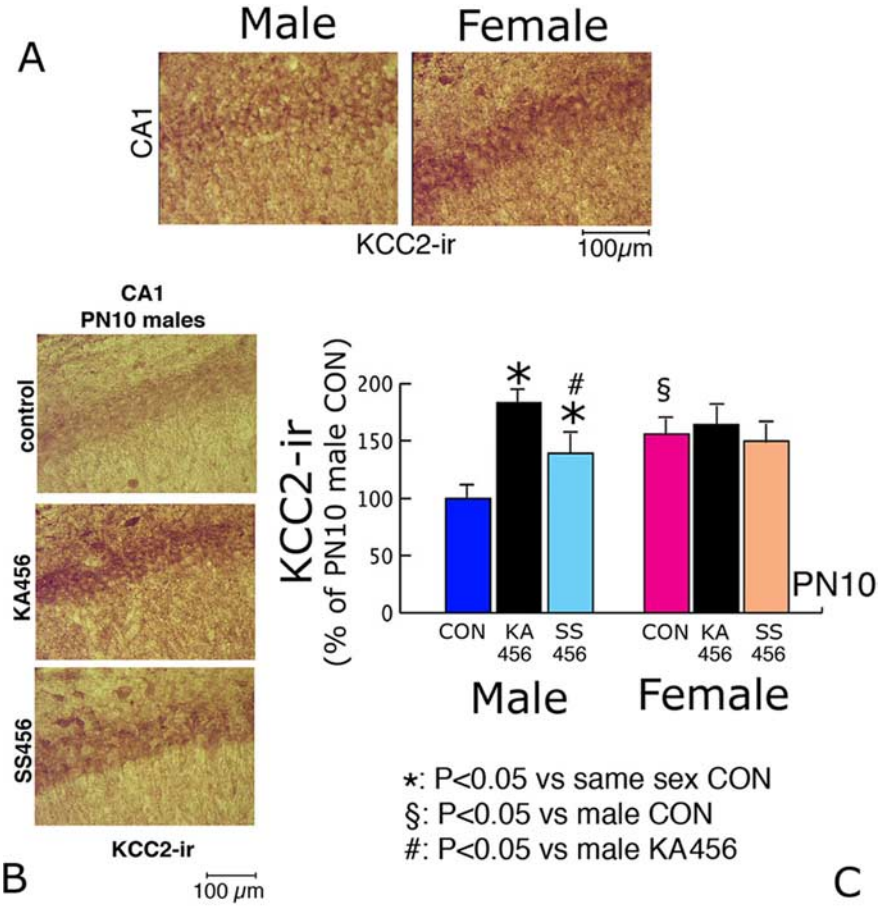

Figure 4. Increased KCC2-ir underlies the negative shift in E_{GABA} in P10 female CON and male KA456 and SS456 groups. ***A***, Male and female rats; representative photos (400× magnification) of CA1 pyramidal neurons from P10 CON rats using a KCC2 specific antibody, counterstained with Novared substrate (brown–red color). Increased KCC2-ir is present in female CA1 pyramidal neurons. ***B***, Male P10 CON, KA456, and SS456 CA1 pyramidal neurons stained with KCC2-specific immunochemistry, using Novared substrate. High levels of KCC2-ir is present in KA456 rats, intermediate in SS456, and low in CON. ***C***, Male and female rats; the results of densitometric analysis of KCC2-ir in CA1 neurons of the three treatment groups are depicted as means ± SEM. Significant sex differences are documented among CON groups. In males only, both KA456 and SS456 have increased KCC2-ir compared with CON (KA456 > SS456 > CON). PN, Postnatal day. Scale bar, 100 μm.

The aberrant re-emergence of depolarizing $GABA_A$ receptor signaling in epileptic tissues is proposed as an important feature of the epileptic state, involved in the generation of interictal and possibly ictal epileptic discharges (Cohen et al., 2002; Khalilov et al., 2003; Sipila et al., 2006). Interestingly, neither male nor female KA456 rats were able to maintain depolarizing $GABA_A$ receptor signaling beyond P14, suggesting that the developmental factors governing E_{GABA} switch were stronger than the effects of 3KA-SE. Moreover, none of the female KA456 rats developed spontaneous seizures in young adulthood. Although more extended monitoring periods may be necessary, if depolarizing $GABA_A$ receptor signaling is a critical component of the epileptic state, the resilience of the immature brain to maintain depolarizing $GABA_A$ergic signaling after 3KA-SE may prevent epilepsy.

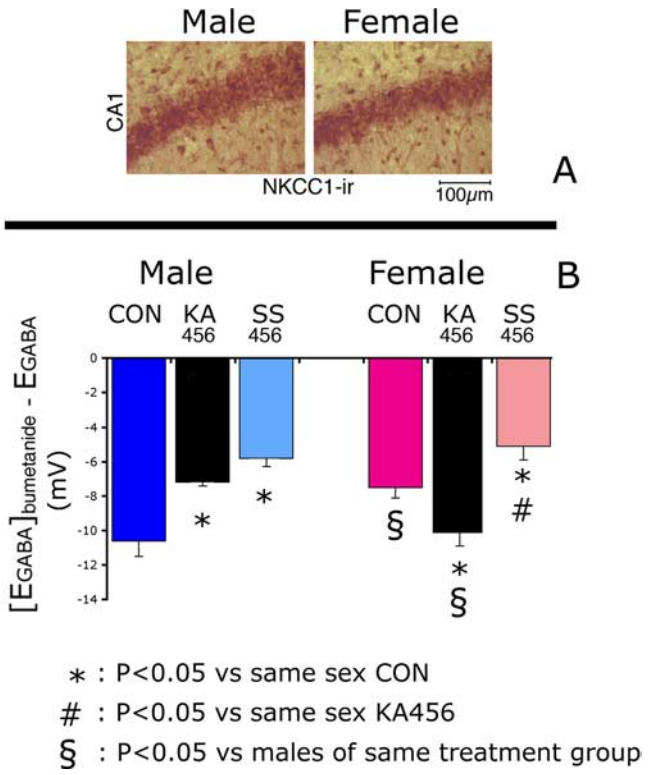

Figure 5. Sex- and treatment-related differences in the activity of bumetanide-sensitive NKCC1-like chloride cotransport in rat P8–14 CA1 pyramidal neurons. ***A***, Male and female rats; representative photos (400× magnification) of the CA1 pyramidal layers of P10 CON rats, stained with NKCC1 specific antibody and counterstained with Novared substrate (brown–red color). No differences are noted in NKCC1-ir between sexes. Scale bar, 100 μm. ***B***, Male and female rats; comparison of $[E_{GABA}]_{bumetanide} - E_{GABA}$ among CON, KA456, and SS456 groups demonstrates significant sex differences in CON and KA456 groups, but not in SS456. In males, the effect of bumetanide on E_{GABA} is smaller in KA456 and SS456 compared with CONs. In females, the effect of bumetanide on E_{GABA} is as follows: KA456 > CON > SS456. Results in ***B*** represent the mean ± SEM.

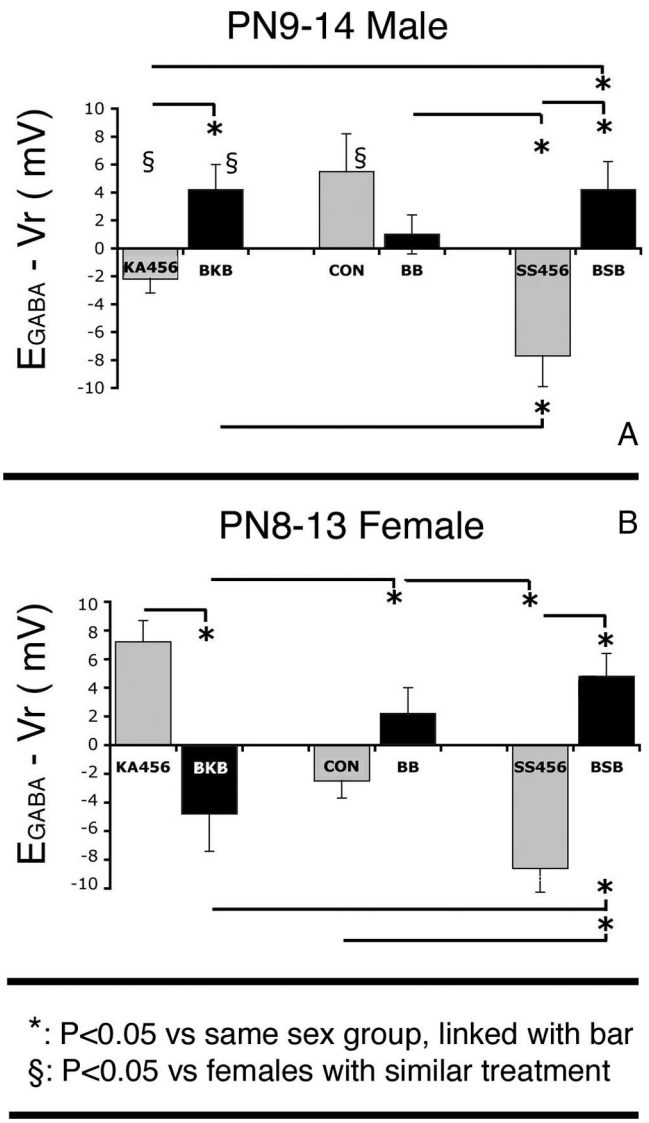

Figure 6. ***A***, ***B***, Effects of bicuculline treatment on $E_{GABA} - V_r$ of P9–P14 male (***A***) and P8–P13 female (***B***) rat CA1 pyramidal neurons. Bicuculline was given 10 min before kainic acid or saline injections and 6 h later at P4, P5, and P6, in both males and females. The bicuculline treatments reversed the directions of $E_{GABA} - V_r$ shifts in the CA1 pyramidal neurons of KA456 and SS456 groups, but their effects were not statistically significant in CON. No sex differences in $E_{GABA} - V_r$ were seen in BB-treated pups. Results represent the mean ± SEM. PN, Postnatal day.

Indeed, in the majority of *in vivo* studies in naive rodents, the risk of developing epilepsy is rare if SE is induced in the first postnatal week and increases with age thereafter (Stafstrom et al., 1992; Moshé, 1993; de Rogalski Landrot et al., 2001; Holmes et al., 2002; Roch et al., 2002; Raol et al., 2006; Suchomelova et al., 2006; Xiu-Yu et al., 2007).

$GABA_A$ receptors are activated by neurosteroids and mediate stress effects (Bianchi et al., 2002; Spigelman et al., 2002). Neonatal stress, including maternal separation, has long-term consequences on brain development, which can be sex specific (Nunez et al., 2000; Hsu et al., 2003; Zimmerberg and Kajunski, 2004; Stamatakis et al., 2006). It is shown here that prolonged maternal separation causes a negative E_{GABA} shift in both sexes, although brief maternal separation did not influence E_{GABA} (Isaeva et al., 2006). The precocious termination of the age-appropriate depolarizing $GABA_A$ signaling may disrupt normal brain development in male, but not in female, CA1 pyramidal neurons, which have already switched at the time of experimentation, leading to sex-specific long-term sequelae.

Maternal separation decreases bumetanide-sensitive chloride transport in both sexes. Low bumetanide concentrations preferentially inhibit NKCC1, decreasing E_{GABA} (Russell, 2000; Dzhala et al., 2005; Banke and McBain, 2006; Reynolds et al., 2007). NKCC1-ir was similar among the groups, suggesting that bumetanide either inhibited other chloride transporters or led to post-translational inactivation of NKCC1. Stress signaling can modulate the activity of NKCC1 by altering its phosphorylation (Darman et al., 2001; Piechotta et al., 2002; Moriguchi et al., 2005; Anselmo et al., 2006; Delpire and Gagnon, 2006; Strange et al., 2006; Vitari et al., 2006). In males only, neonatal separation also increases KCC2-ir, contributing to the negative E_{GABA} shift. This could result from stress-induced $GABA_A$ receptor-mediated depolarization of male CA1 pyramidal neurons (Galanopoulou et al., 2003a) or other sex-specific stress-signaling pathways.

The separation stress-induced decrease in E_{GABA} requires active $GABA_A$ receptors, but is not directed by the depolarizing or hyperpolarizing $GABA_A$ PSCs. One possibility is that chloride gradient around stress-responsive, δ-subunit-containing extrasynaptic $GABA_A$ receptors (Smith et al., 2007) may not differ between male and female rat P4–P6 CA1 pyramidal neurons. In contrast, the observed sex differences in $GABA_A$ PSCs may per-

tain to synaptically located $GABA_A$ receptors, which are strongly activated during seizures. Intracellular differences in chloride concentration and KCC2 expression, both across the axonodendritic processes and around extrasynaptic $GABA_A$ receptors, have been described previously (Gulyas et al., 2001; Duebel et al., 2006; Gavrikov et al., 2006).

Prolonged bicuculline administration abolished the 3KA-SE, stress, and sex-related differences in $E_{GABA} - V_r$, rendering it depolarizing, with single exception the female BKB group. $GABA_A$ receptors may therefore act as sensors and broadcasters of epigenetic (seizures, stress) and sex-related signals implicated in the regulation of E_{GABA}. The negative E_{GABA} shift in BKB female neurons may stem from $GABA_A$-independent pathways activated by 3KA-SE in females. Alternatively, excessive GABA release during seizures may reverse the bicuculline inhibition of $GABA_A$ receptors in females.

In summary, the direction of $GABA_A$ receptor signaling in rat neonatal CA1 pyramidal neurons is sex specific, potentially contributing to their sexual differentiation. Consequently, seizures have sex-specific effects on hippocampal $GABA_A$-related function, distinct from separation stress, with important repercussions for brain development and possibly epileptogenesis.

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