Early Life Status Epilepticus and Stress Have Distinct and Sex-Specific Effects on Learning, Subsequent Seizure Outcomes, Including Anticonvulsant Response to Phenobarbital

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SUMMARY

Aims: Neonatal status epilepticus (SE) is often associated with adverse cognitive and epilepsy outcomes. We investigate the effects of three episodes of kainic acid-induced SE (3KA-SE) and maternal separation in immature rats on subsequent learning, seizure susceptibility, and consequences, and the anticonvulsant effects of phenobarbital, according to sex, type, and age at early life (EL) event. **Methods:** 3KA-SE or maternal separation was induced on postnatal days (PN) 4–6 or 14–16. Rats were tested on Barnes maze (PN16–19), or lithium–pilocarpine SE (PN19) or flurothyl seizures (PN32). The anticonvulsant effects of phenobarbital (20 or 40 mg/kg/rat, intraperitoneally) pretreatment were tested on flurothyl seizures. FluoroJadeB staining assessed hippocampal injury. **Results:** 3KA-SE or separation on PN4–6 caused more transient learning delays in males and did not alter lithium–pilocarpine SE latencies, but aggravated its outcomes in females. Anticonvulsant effects of phenobarbital were preserved and potentiated in specific groups depending on sex, type, and age at EL event. **Conclusions:** Early life 3KA-SE and maternal separation cause more but transient cognitive deficits in males but aggravate the consequences of subsequent lithium–pilocarpine SE in females. In contrast, on flurothyl seizures, EL events showed either beneficial or no effect, depending on gender, type, and age at EL events.

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Introduction

Status epilepticus (SE) consists of prolonged or repetitive seizures without return to baseline that last at least 30 min. The age-adjusted incidence of SE is highest during the first year of life (135 per 100,000 population) [1,2]. Neonatal SE in humans is usually associated with poor neurological outcome and increases the risk of subsequent neurodevelopmental dysfunction and epilepsy [3,4]. Etiology, age, prior seizures or epilepsy, and seizure types at onset are among the factors that determine outcome in humans, as well as in animals, after SE [5–15].

Animal studies support the age-specific effects of SE on a variety of molecular, histopathological, and functional outcomes [7,16]. SE affects the expression, composition, and distribution of GABA_A receptors (GABA_AR) and may disturb the physiology of GABA_ARs in age- and sex-dependent manners [7,17–22]. In prior studies, we focused on the effects of repetitive episodes of neonatal kainic acid-induced SE (3KA-SE) and maternal separation (herein called early life events [EL-events]) upon the direction of GABA_AR signaling (depolarizing vs. hyperpolarizing) in immature rats. We showed that EL-events induced on postnatal days 4–6 (PN4–6) alter the direc-

tion of GABA_AR signaling in an age-, sex-, and region-specific manner. In female hippocampal CA1 pyramidal neurons [17], 3KA-SE on PN4–6 triggers a transient appearance of depolarizing GABA_AR-mediated responses. In contrast, in male hippocampal CA1 pyramidal neurons [17] or substantia nigra pars reticulata (SNR) GABAergic neurons, which have depolarizing GABA_AR signaling on PN4–6, 3KA-SE on PN4–6 caused a precocious emergence of hyperpolarizing responses [17,23]. Maternal separation during the same period causes a premature appearance (male CA1) or augmentation (female CA1) of hyperpolarizing GABA_AR responses [17]. Early cessation of depolarizing GABA_AR signaling may disrupt neuronal differentiation, as shown for the development of excitatory dendritic spines [24–27].

As GABA_ARs in the hippocampus and SNR are closely involved in functions that relate to neuronal excitability, seizure control, and cognitive processing, we investigate here whether EL-events may also have sex- and age-specific long-lasting effects on: (1) susceptibility to subsequent seizures (lithium (Li)–pilocarpine, flurothyl model) [28,29], (2) learning and memory (Barnes maze test) [30], and (3) responsiveness to GABA_AR agonists (e.g., anticonvulsant effects of pentobarbital

in the flurothyl model) [31]. We induced EL-events on PN4–6 as in our previous studies [17,23]. In addition, EL-events were induced on PN14–16 to test whether their consequences on flurothyl seizure thresholds depend upon the developmental age at EL-event induction. PN4–6 is a very immature developmental stage, considered as equivalent to premature neonatal, as indicated by the presence of depolarizing GABA_AR responses in many brain regions (e.g., male CA1 pyramidal hippocampal neurons, male and female GABAergic SNR neurons) [7,17,32]. PN14–16 is considered equivalent to infantile stage, a more advanced stage of brain maturation, including GABA_AR signaling (e.g., male and female GABAergic SNR or CA1 pyramidal neurons have shifted to or are ready to become hyperpolarizing) [1,17,32].

We evaluated learning and memory (PN16–19) and susceptibility to Li–pilocarpine SE (P19) prior to weaning age because: (1) both strongly depend on hippocampal functions, (2) hippocampal GABA_AR signaling has matured to hyperpolarizing in both male and female PN19 rats [17], and (3) identification of factors that worsen SE outcomes at this age is of particular relevance to future epileptogenesis studies, because not all post-SE rats develop epilepsy [33]. Effects on subsequent susceptibility to flurothyl seizures were tested on PN32 to allow comparisons with the previous studies in our laboratory, which had characterized in detail the role of the GABA-responsive neurons of the SNR in flurothyl seizure control [9,31,34,35]. Our results demonstrate sex-specific effects of neonatal 3KA-SE and maternal separation on learning and memory, as well as sex- and EL-event-specific effects on susceptibility to Li–pilocarpine SE. Early life 3KA-SE or separation had no overall impact on subsequent flurothyl seizure thresholds. The only exception was the male rats with 3KA-SE on PN14–16, which exhibited higher thresholds to flurothyl clonic seizures. In contrast, the anticonvulsant effects of phenobarbital were potentiated in an EL-event-, age-, and sex-specific manner.

Materials and Methods

Animals

The offspring from timed pregnant Sprague–Dawley rats (Taconic farms, Inc., Hudson, NY, USA) were used in the experiments. The day of birth was defined as PN0. Rats were maintained at constant temperature (21–23°C) and humidity (40–60%) in a 12 h dark/12 h light cycle with free access to pellet food and water in our animal facility, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. Seizure induction or maternal separation was applied at PN4–6 or PN14–16. During seizure induction or maternal separation, four to six pups were kept together in a cage without the dam at room temperature with no access to food or water for 6 h daily. They were subsequently returned to the dam. Rat pups remained with their mothers until they were weaned at PN21, and they were housed with two to three littermates of the same sex until the end of the experiments. The research protocol was approved by the Institutional Animal Care and Use Committee of the Einstein College of Medicine and was carried out in accordance with the National Institute of Health Guide

for the Care and Use of Laboratory Animals. The experimental design is depicted in Figure 1.

Kainic Acid-Induced Seizures

SE was induced with intraperitoneal (i.p.) injections of kainic acid (KA) in male and female Sprague–Dawley pups between PN4–6 (KA[456]) or PN14–16 (KA[14–16]) on three subsequent days. All seizure inductions were carried out in the afternoon (between 2:00 P.M. and 8:00 P.M.). Pups were kept in groups of five to six pups during seizure induction, in a room with ambient temperature of 27°C. Because incremental doses are necessary in consequent days to develop SE (Galanopoulou [17]), the following doses of KA were used: 1.25 mg/kg at PN4, 1.5 mg/kg at PN5 and 2 mg/kg at PN6 in KA(456) group; 4 mg/kg at PN14, 4.5 mg/kg at PN15, and 5 mg/kg at PN16 in KA(14–16) group. After KA injections, 6 h continuous video monitoring was performed before the pups were returned to the dam to ensure that rats developed SE.

The selected doses of KA induced SE at all age groups. In PN4–6 rats, seizures started 20–30 min after KA injections and consisted of an initial state of immobility followed by hyperactivity and scratching, focal limb myoclonus, and tonic or tonic-clonic seizures lasting at least 5–6 h. At PN14–16, KA induced bouts of scratching followed by episodes of unilateral forelimb clonus and tonic-clonic seizures lasting at least 1.5–2 h. Latency to the first occurrence of seizures was approximately 30 min.

Maternal Separation and Naïve Controls

To control for the maternal separation effect (fasting or stress related), same age and sex pups were vehicle-injected and maternally separated for the same periods as animals subjected to 3KA-SE. Pups were kept in groups of five to six pups during seizure induction, in a room with ambient temperature of 27°C. These groups are called SS(456) and SS(14–16). Pups were kept separate from their dams for 6 h daily under continuous video monitoring. No behavioral seizures were observed in these separated groups. Naïve controls (CON) were kept with their dam during these time periods.

Barnes Maze

Visuospatial learning and memory were evaluated on PN16–19 in KA(456), SS(456), and control groups using the Barnes maze ($n = 7–13$ rats/group) as previously described [36–38] (Figure 1A). The Barnes maze is a validated test often used for the assessment of spatial learning and memory in rodents and exploits the natural inclination of small rodents to escape to a dark environment when placed in an open area under bright illumination. The Barnes maze consisted of an open circular platform 100 cm in diameter. To avoid pups jumping off the table, a paper wall with visual cues was fixed around the periphery of the table. Twelve holes, 5 cm in diameter, were equally spaced around the perimeter of the circle of which only one was open and led to a target dark box. Rats underwent three learning sessions per day during PN16–PN19 (12 learning trials), so that they could recognize and

Figure 1 Experimental design. Panels **A–D** depict the sequence of experimental steps in the various experimental parts of our study. The effects of 3KA-SE or maternal separation on PN4–6 on Barnes maze performance (PN16–19; panel **A**) or Li–pilocarpine SE (PN19; panel **B**) on flurothyl seizures and their response to phenobarbital (PN32, panel **C**) were studied through comparisons with relevant controls. In addition, the effects of 3KA-SE or maternal separation on PN14–16 on flurothyl seizures and their response to phenobarbital (PN32, panel **D**) were also studied in separate studies. KA, kainic acid; SE, status epilepticus.

enter the target hole. In the afternoon of PN19 (fourth day of testing), retention was tested in three sessions. Each learning or retention session terminated when the rat entered the target hole or at 180 seconds, if they failed to find the target hole.

Li–Pilocarpine-Induced SE and Histological Evaluation of SE-Induced Injury

To determine whether neonatal 3KA-SE or maternal separation on PN4–6 alter the susceptibility to Li–pilocarpine-induced seizures, CON, SS(456), and KA(456) male and female rats received Li chloride (3 mmol/kg i.p.; Sigma-Aldrich, St Louis, MO, USA) on PN18 followed by pilocarpine (60 mg/kg i.p.; Sigma-Aldrich) on PN19 [39] (Figure 1B). Rats were monitored for seizures on PN19 for 6 h postinduction. All rats developed SE (continuous seizures for at least 30 min), manifesting a sequence of the following behaviors: scratching, staring, immobility/behavioral arrest (stage 0 [40]), head bobbing (stage 2 [40]) and chewing, unilateral forelimb clonus (stage 3 [40]), bilateral forelimb clonus (stage 4 [40]),

rearing and falling (stage 5 [40]), wet-dog shakes, wild running and jumping (stage 6 [40]), and generalized tonic-clonic seizures (GTCs, stage 7 [40]). Continuous motor seizures (stage 4 or higher) lasted till SE was stopped with pentobarbital (40 mg/kg i.p.) given 2 h after SE onset. Rats were returned to their cage after seizures resolved. Controls received vehicle injections instead of Li chloride or pilocarpine and were monitored for the same periods as rats undergoing SE without access to food or water. A subset of rats, randomly selected, was observed for behavioral seizures the day after (PN20) for 40 min.

SE induced injury was determined 48 h after SE (PN21) based on prior reports showing neuronal injury within 24–48 h post-SE [39,41]. Rats were sacrificed on PN21 by lethal dose of pentobarbital 100 mg/kg i.p. followed by transcardiac perfusion with cold saline and then formalin (10% neutral buffered formalin; Sigma-Aldrich). The following day, brains were transferred in 30% sucrose in phosphate buffered saline and were frozen and stored at -80°C when sunk. Brains were stained with FluoroJade B staining and Nissl staining as in

Galanopoulou et al. (2003) [17,39]. Injury scoring was performed on four to five sections of each rat derived from coronal 40- μ m-thick sections stained with FluoroJadeB that included the anterior dorsal hippocampus. Injury was visually scored and blinded to experimental group, using a variation of scoring systems utilized in Galanopoulou et al. (2003) [39,42]. Sections were scored with a score of 0–3, depending on the extent of area in a specific hippocampal region that contained FluoroJadeB positive cells as follows: score 0: no positive cells; score 1: <30% of area had positive cells; score 2: 31–70% of area had positive cells; and score 3: 71–100% of area had positive cells. Further subdivisions of the scores were used (e.g., 0.25, 0.5, 0.75) to describe more closely the extent of the area with FluoroJadeB positive cells. This scale was utilized as a means to compare the extent of hippocampal regions with injured neurons.

Flurothyl-Induced Seizures and Testing Phenobarbital Effects

On PN32, seizures were induced by flurothyl [bis(2,2,2-trifluoroethyl)ether] (SynQuest Labs, Alachua, FL, USA) inhalation as previously described [31,35,43,44] (Figure 1C,D). Briefly, rats were placed in an airtight chamber (9.34 L), and liquid flurothyl was delivered through a plastic tube at a fixed rate controlled by a pump (40 μ L/min) dripping onto a filter paper in the chamber where it evaporated. In PN32 rats, the flurothyl-induced seizures consisted of clonic followed by tonic–clonic seizures as previously reported [35,43–45]. Rats were exposed to flurothyl until tonic extension of both the forelimbs and hindlimbs was observed or a maximum duration of 20 min was reached. The chamber was flushed with room air for 3 min and cleaned between trials. Flurothyl seizure threshold for the first clonic or tonic seizure was determined as the volume of flurothyl administered until the occurrence of each of these seizure types: threshold for seizure propagation was calculated as the difference between the tonic and clonic thresholds.

To determine whether EL 3KA-SE affects the responsiveness of subsequent seizures to phenobarbital, we evaluated the anticonvulsant effects of two doses of phenobarbital on flurothyl seizure thresholds in male and female KA(456), KA(14–16), SS(456), SS(14–16) groups, and CON. PN32 rats received either phenobarbital (Sigma-Aldrich) (20 or 40 mg/kg) or its vehicle (sterile saline) i.p. 30 min before flurothyl exposure [31].

Statistics

Statistics were performed with JMP10 software (SAS Institute Inc, Cary, NC, USA). ANOVA analysis was performed on the log-transformed latencies for the Li-pilocarpine experiments and flurothyl thresholds, which followed normal distributions. Statistical comparison of the Barnes maze data and flurothyl seizure thresholds performed with a linear mixed model taking into consideration that each rat contributed repeated measures across the different trials. Post hoc intergroup comparisons were performed with Student's *t*-test. Chi-square test was used to compare percentage of rats that exhibit tonic seizures during flurothyl inhalation. Significance was set at $P < 0.05$.

Results

Sex Differences in Effects of 3KA-SE and Maternal Separation at PN4–6 on Visuospatial Learning and Memory

Barnes maze test performed on PN16–19 showed sex-specific differences in performance of rats subjected to three episodes of KA-SE or maternal separation on PN4–6 (Figure 2). Repeated measures analysis revealed significant effects for sex [$F_{\text{sex}}(1, 59) = 7.36$; $P = 0.0087$] and trial number [$F_{\text{trial}}(14, 735) = 88$; $P < 0.0001$] but not EL-event [$F_{\text{EL-event}}(2, 59) = 1.97$; $P = 0.15$]. All groups showed learning, that is decreasing latencies to find the target during the course of the learning sessions.

Males required longer times to find the target hole than females, for the following groups: (1) CON during trials 1 and 2, (2) KA(456) during trials 5 and 6, and (3) SS(456) during trials 2 and 5 ($P < 0.05$, Student's *t*-test).

In KA(456) and SS(456) groups, Barnes maze performance showed sex-specific patterns. In males, both KA(456) and SS(456) groups showed transient delay in learning based on slower completion times during trials 2, 5, and 6 ($P < 0.05$, Student's *t*-test) in KA(456) and trials 4 and 5 in SS(456) ($P < 0.05$, Student's *t*-test) compared with CON. Among females, KA(456) and SS(456) rats showed longer completion times during initial exposure to the task (trials 1 and 2) ($P < 0.05$, Student's *t*-test) but no difference in learning compared with CON. These results revealed that male rats are more vulnerable than females to transient learning delays following neonatal 3KA-SE or maternal separation at PN4–6. No differences were observed on retention sessions among KA(456), SS(456), and control groups.

Effects of Neonatal Seizures or Maternal Separation on Li-Pilocarpine SE on PN19

To determine whether neonatal exposure to 3KA-SE or maternal separation affects susceptibility to subsequent limbic seizures, we exposed CON, KA(456), and SS(456) to Li-pilocarpine SE on PN19. There was a general trend for males to show longer latencies to first clonus than females, but this was significant only in CON (Figure 3A). All rats manifested SE through the duration of 2 h of observation on PN19 and until pentobarbital was given. However, EL-event had sex-specific effects on the incidence of spontaneous seizures the day after Li-pilocarpine SE (PN20). Male KA(456) had no observed spontaneous seizures during the PN20 session ($P = 0.044$ vs. male CON, Fisher's exact test), whereas 85.7% of the female SS(456) rats had motor seizures on PN20 ($P = 0.0128$ vs. female CON, Fisher's exact test) (Figure 3B).

Survival after Li-pilocarpine SE ranged between 25% and 73.33% with deaths occurring either during PN19 SE or on PN20. Female KA(456) rats had significantly reduced survival (25%) than female CON (73.33%) ($P = 0.0069$, Fisher's exact test) (Figure 3C).

We did not find any correlation between seizure scores and mortality. All the rats injected with Li-pilocarpine developed bilateral motor seizures (stage 4 or higher) that were

Figure 2 Effects of early life 3KA-SE and maternal separation on Barnes maze performance of PN16–19 rats. Male and female rats were subjected to either 3KA-SE or maternal separation or no handling during PN4–6 and were subsequently subjected to Barnes maze testing between PN16–19. **(A and B)** Effects on learning trials. Learning trials consisted of three daily sessions on PN16–19. Male KA(456) and SS(456) rats needed longer times than CON to complete trials 2, 5, and 6 and trials 4 and 5, respectively (* $P < 0.05$, Student's t -test; blue for CON; cyan for KA[456]; green for SS[456]). Female KA(456) and SS(456) required longer times to find the target compared to CON at trials 1 and 2 ($P < 0.05$, Student's t -test; red for CON; purple for KA[456]; orange for SS[456]). #: indicates significant differences in completion times between male KA(456) and SS(456) groups ($P < 0.05$, Student's t -test for each comparison). ψ, ω, φ: indicate significant sex differences ($P < 0.05$, Student's t -test) among SS(456), KA(456), and CON groups, respectively. **(C and D)** Effects on retention trials. Retention trials (R1, R2, R3) were performed on PN19. No differences were observed among groups. The graph shows group least square means $\pm$ SEM. KA, kainic acid; SE, status epilepticus.

continuous till the time of pentobarbital injection. 60.56% of rats developed stage 5 or higher seizures (rearing and falling or running or GTCs). Among these, 58.14% died versus 57.14% of those that had only stage 4 seizures. Overall 19.71% of rats developed GTCs after pilocarpine injection (range 0–26.67% per group). Among rats with GTCs, 57.14% died versus 57.9% of those without GTCs.

Histological evaluation for Li-pilocarpine-induced hippocampal injury revealed degenerating neurons in the hilus, dentate gyrus, and CA1 and CA3 pyramidal regions at similar degrees among the various groups. The only statistically significant difference was observed in the CA3 pyramidal region, where female SS(456) rats had more extended injury than the other groups ($P < 0.05$, Student's t -test), which may be attributed to the more protracted seizures (see Figure 3B).

In summary, KA(456) and SS(456) do not alter the latencies to onset of Li-pilocarpine SE. However, they seem to aggravate outcomes in females after PN19 Li-pilocarpine SE, in an EL-event treatment-specific manner. Female KA(456) have higher mortality rates than CON and female SS(456) show higher incidence of spontaneous seizures on PN20 and CA3 pyramidal neuron degeneration on PN21.

The Effects of Early Life 3KA-SE and Maternal Separation on Flurothyl Seizure Thresholds and Incidence of Tonic Seizures on PN32

Figures 4 and 5 show the effects of EL 3KA-SE and maternal separation, as a function of age at induction of SE or separation, on subsequent flurothyl-induced seizures, a model of generalized seizures.

Although EL-events did not have an overall effect on flurothyl seizure thresholds (Figure 4D), intergroup comparisons among saline-pretreated groups demonstrated (1) significantly higher clonic thresholds in male KA(14–16) rats compared to male CON, and male and female KA(456) rats (Figure 4A), and (2) significantly higher propagation thresholds in female KA(14–16) rats compared to male SS(456) or SS(14–16) rats (Figure 4C).

Phenobarbital pretreatment showed specific dose-dependent anticonvulsant effects for both clonic and tonic thresholds, whereas the high dose also reduced the percent of rats that manifested tonic seizures. The majority (93.44–100%) of male and female saline or Pheno-20 injected rats manifested GTCs during flurothyl exposure. Pheno-40 pretreatment, however, prevented

Figure 3 Effects of early life (EL) 3KA-SE (PN4–6) and maternal separation on Li-pilocarpine SE on PN19. Rats were subjected to either 3KA-SE or maternal separation on PN4–6, and Li-pilocarpine SE was induced on PN19. **(A)** Effects on latencies to seizure onset. Latencies to first forelimb clonus were significantly affected by sex ($F_{sex}(1, 89) = 4.63$, $P = 0.034$; ANOVA on log-transformed latencies) but not by EL treatment group ($F_{EL-event}(2, 89) = 0.046$, $P = 0.96$). Males tended to have longer latencies to first clonus, but this reached significance in CON only ($P < 0.05$, Student's t -test). Results are expressed as least square means $\pm$ SEM. **(B)** Effects on seizure occurrence on PN20. A random subset of rats were observed on PN20 (i.e., day after PN19 Li-pilocarpine SE) for seizures. Among males, KA(456) manifested no spontaneous seizures on PN20 in contrast to the other groups ($P = 0.044$ vs. CON, Fisher's exact test). Among females, SS(456) rats manifested more commonly spontaneous seizures (six of seven rats, 85.7%) than female CON (2 of 11, 18.2%) ($P = 0.0128$, Fisher's exact test). **(C)** Effects on survival after Li-pilocarpine SE. Survival till PN21, after PN19 Li-pilocarpine SE, ranged between 25% and 73.33% across groups. The only statistical significant difference was observed between female CON (73.33%) and female KA(456) (25%) ($P = 0.0068$, Fisher's exact test). **(D)** Effects on Li-Pilocarpine SE-induced hippocampal injury on PN21. On PN21, the distribution of FluoroJadeB stained cells in the hippocampus was comparable across groups, with only exception the increased number of FluoroJade B stained cells in the CA3 pyramidal region of female SS(456) rats compared to all other male or female groups, with single exception the male CON ($P < 0.05$, Student's t -test). The numbers of rats studied are depicted on the bars (A–C) or at the legend (D). * $P < 0.05$ between linked groups (A–C) or between female SS(456) and all other groups except for male CON (D: CA3). **(E)** FluoroJadeB staining in the hippocampus of female rats 48 h after Li-pilocarpine SE. The panels present examples of FluoroJade B stained cells (green) in the hilus and CA3 pyramidal region of female CON, KA(456), and SS(456) rats. All groups showed injury at the hilus, but the SS(456) females showed more injury at the CA3 pyramidal layer. KA, kainic acid.

Figure 4 The effects of early life (EL) 3KA-SE or maternal separation on the anticonvulsant effects of phenobarbital on flurothyl-induced seizures in PN32 rats. Pretreatment with phenobarbital increased the flurothyl thresholds for clonic, tonic seizures and seizure propagation in a dose-dependent manner. Statistics were performed on log-transformed thresholds. **(A)** Effects of phenobarbital pretreatment on clonic seizure thresholds. Phenobarbital pretreatment showed a significant effect: $F_{pheno}(2, 290) = 239.5$, $P < 0.0001$. Neither sex nor EL treatment had a significant effect. Both doses of phenobarbital exhibited significant dose-dependent anticonvulsant effects. Saline-injected male KA(14–16) group had higher thresholds than male CON or KA(456) or female KA(456). The anticonvulsant effect of Pheno-40 was more pronounced in male SS(14–16) rats compared to Pheno-40 male CON or KA(456) and female CON. **(B)** Effects of phenobarbital pretreatment on tonic seizure thresholds. Phenobarbital pretreatment showed a significant effect: $F_{pheno}(2, 234) = 390.5835$, $P < 0.0001$ and a significant number of phenobarbital pretreated rats failed to exhibit tonic seizures (see also Figure 5). Neither sex nor EL treatment had a significant effect. The anticonvulsant effect of Pheno-20 was greater in female KA(456) rats compared to female CON, SS(456) and KA(14–16). Statistical significance could not be documented in the Pheno-40 KA(456) pretreated male group because only one rat manifested tonic seizure. **(C)** Effects of phenobarbital pretreatment on seizure propagation thresholds. Both Pheno-20 and Pheno-40 increased seizure propagation thresholds compared to saline-pretreated rats, in all groups, except for male Pheno-40 KA(456) rats: only one exhibited tonic seizure and statistical significance could not be documented. Dose-related differences could not be documented. Female saline-injected KA(14–16) rats exhibited higher propagation thresholds compared to male SS(14–16) and SS(456) rats. **(D)** Effect tests for EL-event, phenobarbital pretreatment, and sex for the clonic, tonic, and propagation thresholds. Data are displayed as least square means $\pm$ SEM. *: indicate significant differences between bar-linked groups ($P < 0.05$, Student's *t*-test). #: indicate significant differences compared to saline-injected same-sex rats that received the same neonatal treatment ($P < 0.05$, Student's *t*-test). 0: indicate significant dose-related differences between same-sex rats (Pheno-20 vs. Pheno-40 pretreated) that received the same neonatal treatment ($P < 0.05$, Student's *t*-test). §: indicate significant sex differences between same EL-event Pheno-20 groups ($P < 0.05$, Student's *t*-test). KA, kainic acid; SE, status epilepticus.

Figure 5 The effects of early life (EL) 3KA-SE or maternal separation on the ability of phenobarbital to prevent flurothyl-induced tonic-clonic seizures in PN32 rats. All saline or Pheno-20 pretreated PN32 rats exhibit tonic-clonic seizures during the 20 min of flurothyl inhalation. In contrast, Pheno-40 pretreatment partially prevents the expression of flurothyl-induced tonic-clonic seizures. This anticonvulsant Pheno-40 effect is statistically significant in the male KA(456), female SS(456) groups, as well as in the KA(14-16) and SS(14-16) male and female rats when compared with either saline- or Pheno-20 pretreated rats of the same sex and EL treatment group ($P < 0.05$, Fisher exact test, two-tailed). Among the Pheno-40 pretreated rats, only male KA(456) appeared significantly more protected than CON from manifesting tonic-clonic seizures ($P < 0.05$, Fisher exact test, two-tailed). (*): indicate $P < 0.05$ versus saline or Pheno-20 same-sex and same group rats. (#): indicates $P < 0.05$ versus linked groups. KA, kainic acid; SE, status epilepticus.

tonic seizures during the 20 min window of flurothyl testing, in a significant number of rats. This effect was significant, compared to respective CON or Pheno-20 groups, for the following: KA(14-16) and SS(14-16) of both sexes, male KA(456) and female SS(456) groups. The Pheno-40 pretreated male KA(456) group, in particular, showed the lowest percentage of rats with tonic seizures ($P < 0.05$ vs. male and female Pheno-40 CON, Fisher's exact test, two-tailed) (Figure 5). Phenobarbital pretreatment (Pheno-20 or Pheno-40) also increased the propagation thresholds compared to saline-pretreated groups in all groups except for male Pheno-40 KA(456), as only one rat manifested tonic seizure. Dose dependency could not be documented. Overall, these suggest that EL 3KA-SE or separation do not impair the anticonvulsant effects of phenobarbital on flurothyl tonic seizures, but in certain situations, they potentiate them (e.g., male KA[456]). Only the following exceptions were observed. For clonic thresholds, statistical significance could not be documented between Pheno-20 and saline-pretreated male SS(456) rats (Figure 4A). For tonic thresholds, statistical significance on thresholds could not be tested in Pheno-40 male KA(456) rats because only one rat manifested tonic seizures.

In summary, EL exposure to 3KA-SE on PN14-16, but not on PN4-6, delays the onset of clonic seizures in KA(14-16) male rats on PN32, suggesting that the age of 3KA-SE exposure may have specific effects on the maturation of networks that control clonic seizures. Furthermore, EL-events do not impair the anticonvulsant effects of phenobarbital on flurothyl seizures on PN32, but rather potentiate them in specific groups: male KA(456) (less tonic seizures in Pheno-40 rats), female KA(456) (increased tonic thresholds in Pheno-20 rats), and male SS(14-16) (increased clonic thresholds in Pheno-40 rats).

Phenobarbital Preretreatment Effects on Survival Rates After Flurothyl Seizures in PN32 Rats

Survival rates were significantly increased by Pheno-40 pretreatment in both sexes although this effect was not statistically significant in SS(456) rats (Figure 6). Among Pheno-20 groups, only female KA(456) rats had significantly higher survival rates than saline-injected KA(456) rats. Furthermore, female SS(456) had higher survival rates than males in both saline- or Pheno-20 pretreated rats.

Conclusion

We show that neonatal 3KA-SE or intermittent maternal separation on PN4-6 have sex-specific effects on learning and memory but no significant effects on the latencies or thresholds to first clonic seizure in the Li-pilocarpine model (PN19) or flurothyl seizures (PN32). However, they tend to produce sex- and treatment-specific effects on the incidence of spontaneous seizures the day after Li-pilocarpine SE, the resultant hippocampal injury and mortality. In general, the age at EL-event does not alter thresholds to clonic or tonic flurothyl seizures on PN32, with single exception the male KA(14-16) group that had higher clonic thresholds. Despite the more severe seizures in both male and female KA(456) compared to KA(14-16) rats, only male KA(14-16) rats showed higher clonic thresholds than KA(456). In the flurothyl seizure model, phenobarbital pretreatment showed dose-dependent anticonvulsant effects in all groups and protected from mortality, following sex- and EL-event-specific patterns. These results suggest that EL-events (3KA-SE, maternal separation) modify the

Figure 6 Survival rates after PN32 flurothyl seizures: effects of phenobarbital pretreatment as a function of sex and early life (EL)-event. Early life 3KA-SE or maternal separation does not affect survival after flurothyl seizures in PN32 rats. Pheno-20 groups did not have significant different survival rates from saline-injected rats, with only exception the male SS(14–16) and female KA(456) which had higher survival rates than saline-injected same EL-event groups. Pheno-40 pretreatment conferred complete protection (100% survival) in all groups except for male CON, although this increase was not statistically significant for SS(456) groups. Sex differences in survival were observed between saline-injected and Pheno-20 pretreated SS(456) groups (higher survival rates in females). # $P < 0.05$ versus saline-injected same-sex and EL-event group (Fisher's exact test). * $P < 0.05$ between linked groups (Fisher's exact test). KA, kainic acid; SE, status epilepticus. The numbers below each bar indicate the number of rats tested in each experimental group.

outcomes of subsequent seizures in a model, sex, EL-event, and age-at-induction-specific manner.

Effects on Learning on PN16–19

We found sex-specific patterns of learning and influences of EL-events, although learning was eventually achieved in all groups. Among CON, males showed longer completion times of the first trials than females, without further differences in learning. KA (456) or SS(456) males had transient deceleration of learning. Female KA(456) and SS(456) rats, in contrast, showed slow onset of learning but normal learning acquisition. These sex and experience-related differences in learning are likely due to specific effects on modifiers of learning (e.g., anxiety) or sex-specific learning strategies (e.g., stimulus-response learning in prepubertal males) [46]. Following various maternal separation protocols, separated males have also been reported to have more learning deficits, impaired spatial recognition, more anxiety-like behavior, less inhibitory avoidance, and worse object recognition than females [47–51]. Long-term deficits in learning and memory have been demonstrated for immature rats subjected to recurrent, brief neonatal seizures, or experimental hyperthermic seizures although sex differences have not been described [52,53].

Effects on Subsequent Seizure Thresholds

We did not find sex differences in flurothyl seizure thresholds of CON, in agreement with Velisek et al. [43]. However, in the Li-pilocarpine model, PN19 males had longer latencies to first clonic seizure than females (significant only in CON). This was due to a subset of males with longer latencies, suggesting a role of genetic or other individual susceptibility factors, which could delay the maturation of the networks controlling limbic seizures in males [reviewed in Ref. 34]. Slower maturation of regions involved in seizure expression and control (e.g., hippocampus or SN) has indeed been described in developing male rats

[17,32,34,35]. In support of the immaturity hypothesis, our previous study of young adult rats subjected to Li-pilocarpine SE did not yield any differences in latencies to first clonus between males and ovariectomized females [39]. In contrast, shorter latencies and higher incidence of full limbic seizures have been reported in adult male rats exposed to Li-pilocarpine SE compared to females [54,55], but these studies were not controlled for the different hormonal environments during the estrous cycle phases [54].

Early life SE or maternal separation had no significant effect on latencies or thresholds to seizures, in either model, except that male KA(14–16) rats—but not KA(456)—had increased thresholds to flurothyl clonic seizures. This suggests that age at 3KA-SE may be important for outcomes, at least in males, although it is also possible that the higher clonic thresholds may be a transient effect due to the shorter (2 week long) latency period between KA(14–16) and flurothyl testing. Further studies comparing flurothyl seizure thresholds 2 versus 4 weeks after 3KA-SE or separation induction would be useful to differentiate the two possibilities. Maternal separation (PN2–14, 3 h daily) reduced thresholds to kindling in adult females [56] and accelerated kindling in both sexes [57]. In contrast, PN2–9 maternally separated rats had similar pentylenetetrazole seizure threshold compared to controls on PN100 [58]. In summary, these studies indicate that a variety of factors may determine outcomes after early maternal separation, including age when this occurs, period till subsequent seizure testing, sex, and possibly the type of seizure tested.

Effects on the Consequences of Subsequent Li-Pilocarpine SE

We found significant sex and EL-event-specific effects on the consequences of subsequent Li-pilocarpine SE. Male KA(456) rats had lower incidence of spontaneous motor seizures the day after Li-pilocarpine SE, whereas mortality or ensuing hippocampal injury were unaffected. Among females, SS(456) had higher incidence of spontaneous motor seizures on PN20 than CON. PN20

seizures may reflect either unresolved SE or seizure recurrence. In support of persisting SE, previous studies from our laboratory using prolonged EEG monitoring after KA-SE in PN15 rats, demonstrated persistence of electrographic SE for at least 16 h after KA administration [59]. It is therefore likely that the higher rate of PN20 motor seizures represents longer and/or more severe SE in the SS(456) females. This is further corroborated by the higher injury scores in the CA3 area in this group. Although video-EEG studies were not performed in the current study, to avoid the additional variable of surgery, future studies with video-EEG monitoring would be helpful to evaluate the duration and severity of SE in the various groups.

Mortality was increased in female KA(456) rats after Li-pilocarpine SE. The lack of increase in PN20 seizures or SE-induced injury in the female KA(456) rats could therefore reflect a survival selection bias, whereby the most severely affected rats have already died.

Thus, EL-events render female rats more vulnerable to the consequences of subsequent SE than males, as far as seizure persistence and resultant hippocampal injury (in SS[456] females) or mortality (in KA[456] females) are concerned. These findings also highlight that changes in seizure thresholds do not predict seizure persistence, ensuing hippocampal injury or mortality and may not be sufficient independent biomarkers of epileptogenesis. Selective vulnerability of immature female rats to EL stressors has also been shown by others. Maternally separated (PN2–14, 3 h daily) female rats were kindled faster than males in adulthood, using the amygdala kindling model [57,60], and had more CA3 pyramidal layer injury postkindling [57]. In contrast, shorter maternal separation periods (PN4–5, 1 h daily) had no effect on hippocampal kindling, although it is unclear if this was due to the milder EL stressors [61].

It has been proposed that maternal separation increases subsequent seizure susceptibility by predisposing them to secrete more corticosterone during seizure induction [57]. The higher vulnerability of KA(456) and SS(456) females to Li-pilocarpine SE may be due to accelerated maturation of networks involved in seizure control, rendering them as vulnerable to SE as older females normally are. We had showed, for example, that SS(456) induced an earlier appearance or enhancement of hyperpolarizing GABA_AR signaling in the CA1 pyramidal neurons of the hippocampus [17] and the SNR [23]. To clarify whether the apparent sex-specific vulnerability reflects observations at different timepoints of the sex-specific maturation curves, comparisons of the effects of EL-events on the consequences of SE induced at different ages would be useful.

Effects on Phenobarbital Responsiveness in the Flurothyl Model

Flurothyl is a known GABA_AR antagonist [62]. Phenobarbital pretreatment showed a clear dose-related anticonvulsant effect for both clonic and tonic flurothyl seizures. The high phenobarbital dose (Pheno-40) also protected against flurothyl seizure-induced mortality. Anticonvulsant effects of the lower dose of phenobarbital were also shown in a prior study, but only against tonic-clonic seizures [31].

The anticonvulsant effects of phenobarbital against flurothyl tonic seizures were more pronounced in KA(456) rats. Fewer

male Pheno-40 KA(456) rats developed tonic seizures, whereas female Pheno-20 KA(456) rats had increased flurothyl tonic seizure thresholds. These findings indicate that KA(456)—but not KA(14–16)—may trigger a long-lasting increase in the expression of phenobarbital sensitive GABA_AR subunits (e.g., β subunits) in regions that need to be silenced to inhibit flurothyl-induced tonic seizures. In agreement, our preliminary unpublished studies show an increase in β 2 GABA_AR subunit in the anterior SNR of male KA(456) rats. The observed sex- and EL-event-related differences in phenobarbital sensitivity emphasize the presence of distinct and complex GABA_AR-sensitive networks controlling clonic and tonic seizures that are differentially altered by the prior life experiences as well as the age when these occur. Other studies have indicated that the age at first SE may define the long-term effects on the expression of specific GABA_AR subunits in the hippocampus [16,18,19].

The highest dose of phenobarbital (Pheno-40) exhibited the greatest protection against flurothyl seizure mortality in almost all groups. Interestingly, female SS(456) rats were less vulnerable to flurothyl seizure-related death, in both saline and phenobarbital-treated groups, which was independent of any anticonvulsant effects. It is therefore possible that early maternal separation on PN4–6 may cause sex-specific and long-lasting priming beneficial effects on brain regions controlling cardiovascular or respiratory functions that protect from seizure-induced mortality.

Effects of 3KA-SE versus Maternal Separation

The type of EL-event played no significant effect on the latencies or thresholds to Li-pilocarpine or flurothyl seizures. In contrast, EL-events influenced the anticonvulsant effects of phenobarbital on tonic flurothyl seizures (drug effect was potentiated in KA [456] females). Female SS(456) rats exhibited more hippocampal injury than KA(456) although this could be due to selection of survivors, given the high mortality in the KA(456) female group.

In summary, our study demonstrates that EL-events have different impact on learning and subsequent seizure susceptibility or consequences. Among animals that experienced neonatal 3KA-SE or maternal separation, a sex-specific vulnerability to learning deficits or seizures was observed. Males were more vulnerable to transient learning delays, which would be amenable to modifications of learning strategies. In contrast, females were more vulnerable to the consequences of subsequent Li-pilocarpine SE (mortality or seizure persistence and hippocampal injury). Phenobarbital maintained its anticonvulsant and protective effects in all groups, in the flurothyl model. Enhanced anticonvulsant phenobarbital effects on flurothyl tonic seizures were seen in male and female KA(456) rats. Neonatal maternal separation in females (SS [456]) also resulted in a beneficial priming effect reducing mortality from subsequent flurothyl seizures, independent of any effects on seizures. It would be interesting to investigate possible long-lasting alterations of cardiovascular or respiratory control networks, by early maternal separation in females. These findings indicate that the molecular and functional consequences of EL experiences may have distinct and often dissociated consequences on cognitive and seizure outcomes that are further modified by gender, type, and age at EL-event, as well as the type of subsequent seizure.

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Conflict of Interest

The authors declare no conflict of interest.

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