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·Original Article·

One hour of pilocarpine-induced status epilepticus is sufficient to develop chronic epilepsy in mice, and is associated with mossy fiber sprouting but not neuronal death

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ABSTRACT

Determining the minimal duration of status epilepticus (SE) that leads to the development of subsequent spontaneous seizures (i.e., epilepsy) is important, because it provides a critical time-window for seizure intervention and epilepsy prevention. In the present study, male ICR (Imprinting Control Region) mice were injected with pilocarpine to induce acute seizures. SE was terminated by diazepam at 10 min, 30 min, 1 h, 2 h and 4 h after seizure onset. Spontaneous seizures occurred in the 1, 2 and 4 h SE groups, and the seizure frequency increased with the prolongation of SE. Similarly, the Morris water maze revealed that the escape latency was significantly increased and the number of target quadrant crossings was markedly decreased in the 1, 2 and 4 h SE groups. Robust mossy fiber sprouting was observed in these groups, but not in the 10 or 30 min group. In contrast, Fluoro-Jade B staining revealed significant cell death only in the 4 h SE group. The incidence and frequency of spontaneous seizures were correlated with Timm score (P = 0.004) and escape latency (P = 0.004). These data suggest that SE longer than one hour results in spontaneous motor seizures and memory deficits, and spontaneous seizures are likely associated with robust mossy fiber sprouting but not neuronal death.

Keywords: epileptogenesis; pilocarpine; Fluoro-Jade B staining; Timm staining; Morris water maze

INTRODUCTION

Acquired epilepsy is a devastating neurological disorder characterized by an initial brain insult and the subsequent development of recurrent seizures, which disrupt normal brain functions and affect the quality of life. Several insults, such as neonatal hypoxia, febrile seizures/hyperthermia, head trauma, stroke and brain tumors can cause brain injury and the development of spontaneous seizures after a latent period[1]. However, the exact underlying mechanisms of acquired epilepsy remain unclear, which are suggested to involve neuronal death, axonal sprouting and synaptic reorganization, and ion channelopathy[2-4].

One commonly-used animal model of acquired epilepsy is established by the chemoconvulsant pilocarpine, a muscarinic receptor agonist, to induce repeated acute seizures (status epilepticus, SE) and cause initial brain damage. In this model, animals are injected with pilocarpine and allowed to experience SE for 90 min to 3 h, which leads to consistent chronic seizures[5,6]. Clinically, it is important to intervene and stop seizures as soon as possible to minimize the subsequent pathological changes in the brain, thus preventing the development of epileptogenesis and brain damage. The current study aimed to determine the minimal duration of SE that leads to the development of epilepsy, and provide a time-window for optimal intervention in SE.

MATERIALS AND METHODS

Chemicals

Pilocarpine, scopolamine hydrobromide, Fluoro-Jade B (FJB)

and gum arabic were from Sigma (St. Louis, MO). Diazepam was from Jiangxi Pharmaceuticals, Ltd (Nanchang, China).

Animals and Seizure Monitoring

The animal protocol was in accord with the guidelines of Zhejiang University Animal Care and Use Committee (SYXK 2012-0178). Male ICR (Imprinting Control Region) mice (25–30 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (certificate: SCXK 2007-0005) and raised at 24 ± 1°C with 40–60% humidity at a 12-h light/dark cycle. Mice were injected intraperitoneally with 2% pilocarpine (100 mg/kg) to induce SE. To antagonize peripheral muscarinic action, scopolamine hydrobromide (0.5 mg/kg) was injected 30 min prior to pilocarpine administration. In control animals, pilocarpine was replaced with saline. Seizure severity was rated by the Raccine scale[7]: category 1, immobility and facial twitch; category 2, head nodding; category 3, forelimb clonus; category 4, rearing; and category 5, rearing and falling. The onset of SE was defined as the beginning of category 4–5 seizures. SE was terminated by 0.1% diazepam (10 mg/kg) after 10 min, 30 min, 1 h, 2 h or 4 h. If the animals did not develop category 4–5 seizures 30 min after pilocarpine injection, an additional injection at 25% of the original dose was given, until they developed stage 4–5 seizures. If the animals did not develop stage 4 seizures after 3 additional applications of pilocarpine, they were excluded from the subsequent study.

From the second day on, each mouse with SE was video-monitored for spontaneous seizures for 10 h/day for 60 days. Videotapes were reviewed by observers blinded to the model. Video monitoring was stopped if the mouse had a motor seizure of stage 4 or greater.

Tissue Preparation

Seven (for FJB staining) or 28 days (for Timm staining) after pilocarpine (or saline) injection, mice were anesthetized with 10% chloral hydrate (1 g/kg, i.p.) and perfused with 0.1 mol/L phosphate buffered solution followed by 4% paraformaldehyde (PFA). For Timm staining, sodium sulfide was perfused before PFA as described previously[8]. The brains were removed and fixed in 4% PFA overnight, then transferred to 30% sucrose solution, and kept at 4°C. Frozen coronal sections were cut at 20 μm on a microtome (Microm HM525, Thermo Scientific, Waltham, MA). Five sections selected from a one-in-six series were collected from each animal at the same level of the hippocampus, starting at 2.8 mm posterior to bregma, and used for subsequent staining.

FJB Staining

FJB staining was performed as described previously[9]. Briefly, sections were first immersed in 1% NaOH/80% ethanol for 5 min and then in a sequence of 70% ethanol, 50% ethanol and distilled water for 2 min. After incubation in 0.06% potassium permanganate for 10 min, the sections were rinsed gently and stained with 0.0004% FJB in 0.1% acetate for 20 min in the dark. A Carl Zeiss LSM Pascal confocal microscope with a 10×/0.3 numerical aperture objective was used to acquire images (920 × 920 μm^2 fields) in CA1, CA3, and the dentate hilus at a similar location in different animals. The numbers of FJB-positive cells per image field in CA1, CA3, and hilus were counted in each of five sections per animal.

Timm Staining

Slices were incubated in a solution containing 50% gum arabic, 60 mL; citric acid buffer, 10 mL; 5.67% hydroquinone solution, 30 mL; 17% silver nitrate, 0.5 mL in the dark for ~120 min. Mossy fiber sprouting in the molecular layer of the dentate hilus was assessed under a Nikon light microscope (Tokyo, Japan). The degree of mossy fiber sprouting was rated using semi-quantitative analysis[8,10] as follows: (1) sparse Timm granules in the supragranular zone; (2) more numerous granules in a continuous distribution; (3) prominent granules and patches; (4) dense laminar band in the supragranular layer; and (5) dense laminar band extending to the inner molecular layer.

Morris Water Maze Test

Mice were trained in the Morris water maze for four consecutive days (four trials per day at ~1-h intervals) after 2 months of video monitoring. The mice were first placed on the platform for 10 s, then randomized to the four quadrants. Recordings were stopped 10 s after the mouse reached the platform (60 s maximum for each quadrant). The mouse was directed to the platform if it did not find it within 60 s, and was allowed to stay there for 10 s. On the fifth day, each mouse was placed in the quadrant diagonally opposite the previous platform location and the time to reach the location was recorded as the escape latency. Swimming distance, swimming speed and the number of target quadrant crossings were also analyzed.

Statistics

Results are presented as mean ± SEM. Differences among experimental groups were analyzed by one-way ANOVA with Dunnett's test for *post-hoc* comparison (version 10.0, SPSS Inc., Chicago, IL). Correlations among seizure incidence, mossy fiber sprouting, neuronal cell death and memory deficit were analyzed by the Spearman rank correlation method. P <0.05 was considered to be statistically significant.

RESULTS

Pilocarpine-Induced SE and Mortality Rate of Mice

Seizures first occurred at 15–30 min after pilocarpine injection, progressed to continuous category 4–5 seizures (i.e., SE), and were terminated at each specific time point by diazepam. Some animals died on days 2–3, and the mortality rate appeared to increase with the prolongation of SE, with no deaths in the 10-min SE group and the highest mortality in the longest (4 h) SE group (Table 1).

Table 1. Effect of SE duration on survival rate

Duration of SE	Total No.	No. surviving mice	Survival rate (%)
Control	15	15	100
10 min	15	15	100
0.5 h	22	20	90.9
1 h	22	19	86.4
2 h	18	14	77.7
4 h	19	13	68.4

One Hour of SE Was Sufficient to Develop Epilepsy, and the Frequency Increased with SE Duration

Recurrent spontaneous motor seizures started to occur ~1 week after SE, with typical tonic-clonic manifestation and generally lasting no more than 1 min. The incidence of spontaneous motor seizures, latency to the first seizure and seizure frequency are summarized in Table 2. The results

Table 2. Effect of SE duration on spontaneous seizures

SE duration	Seizure incidence (%)	Seizure latency (days)	Seizure frequency (per day)
Control	0.0	0.0	0.0
10 min	0.0	0.0	0.0
0.5 h	0.0	0.0	0.0
1 h	21.1	8.4±3.2	2.1±1.2
2 h	57.1	7.9±2.8	3.5±1.7
4 h	76.9	7.6±3.1	4.6±1.9

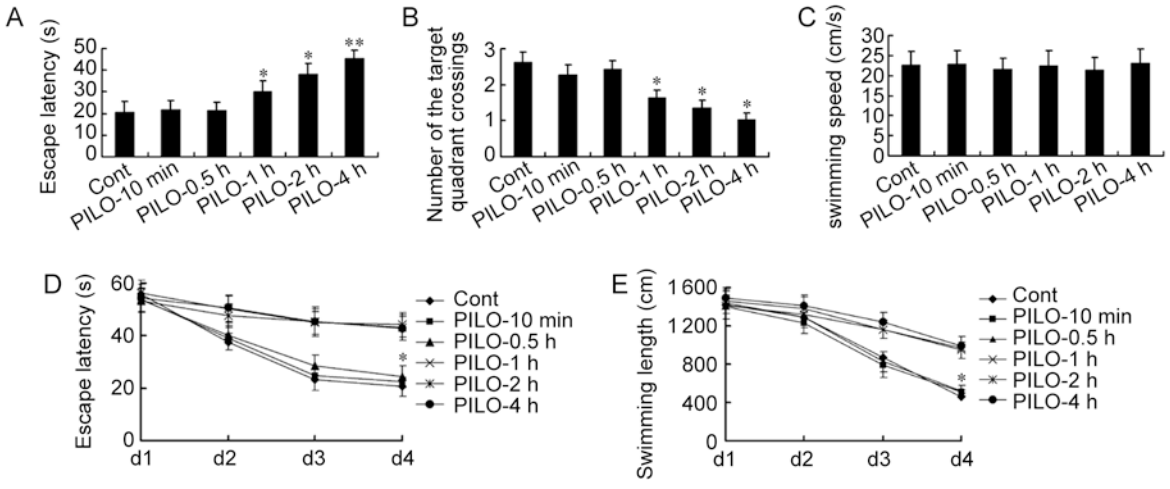

Fig. 1. Prolonged SE induced behavioral changes in the Morris water maze. Mice were trained for four consecutive days after 60 days of monitoring. A: Escape latency on day 5 increased in the groups with SE for 1 h or longer. B: Number of target quadrant crossings on day 5 decreased in the groups with SE for 1 h or longer. C: Swimming speed on day 5 did not differ among groups. D and E: Mean latency to reach the platform and mean swimming distance during the four consecutive training days (n = 13–20 mice per group, *P <0.05, **P <0.01 compared to control group, one-way ANOVA). Cont, control; PILO, pilocarpine.

show that SE lasting for 1 h is sufficient to cause chronic spontaneous motor seizures, and their incidence and frequency increased with SE duration.

One Hour SE Caused Spatial Memory Impairment in Mice

The Morris water maze was used to assess the effect of SE and spontaneous seizures on spatial memory. SE for 10 min and 0.5 h did not influence the escape latency compared to control (Fig. 1). However, SE for 1 h or longer markedly increased the escape latency (Fig. 1A), decreased the number of target quadrant crossings (Fig. 1B), and the severity aggravated with SE duration. No significant difference was found in swimming speed (Fig. 1C). SE for 1 h or longer also induced significant impairment during training (Fig. 1D, E).

SE less than 4 h Did Not Cause Hippocampal Neuronal Death Regardless of the Development of Spontaneous Motor Seizures

Potential neuronal death in the hippocampus was assessed by FJB staining that labels dying neurons. In six out of 10 mice that experienced prolonged SE (4 h), a significant

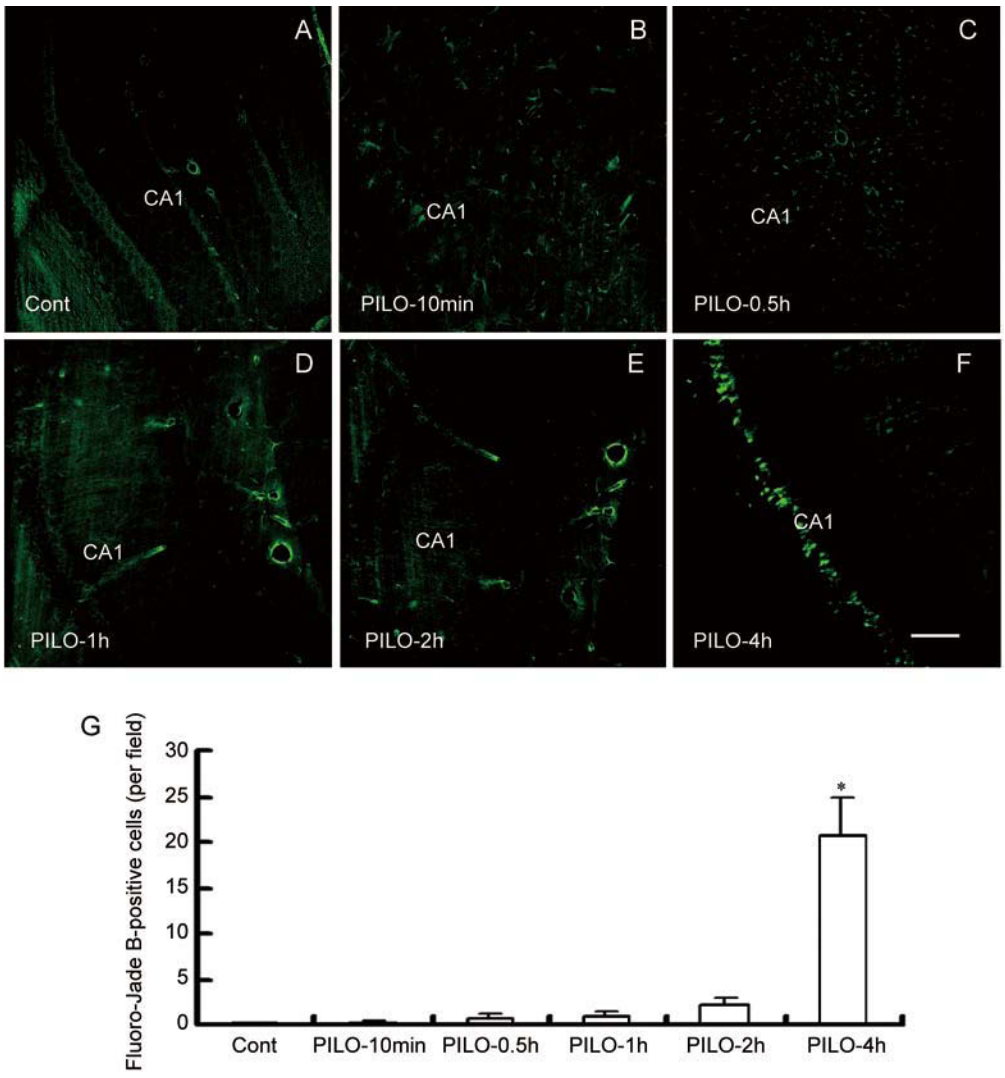

Fig. 2. Prolonged SE induced neuronal death in hippocampal CA1. A–F: Representative sections stained with Fluoro-Jade B in the hippocampus from mice with variable durations of SE. Abundant Fluoro-Jade B-positive neurons were present only in the 4-h SE group. Scale bar, 200 μm. G: Quantitative analysis demonstrated a significant increase in Fluoro-Jade B-positive neurons in the 4-h SE group compared to the other groups (*n* = 10 mice per group, **P* <0.01, one way ANOVA). Cont, control; PILO, pilocarpine.

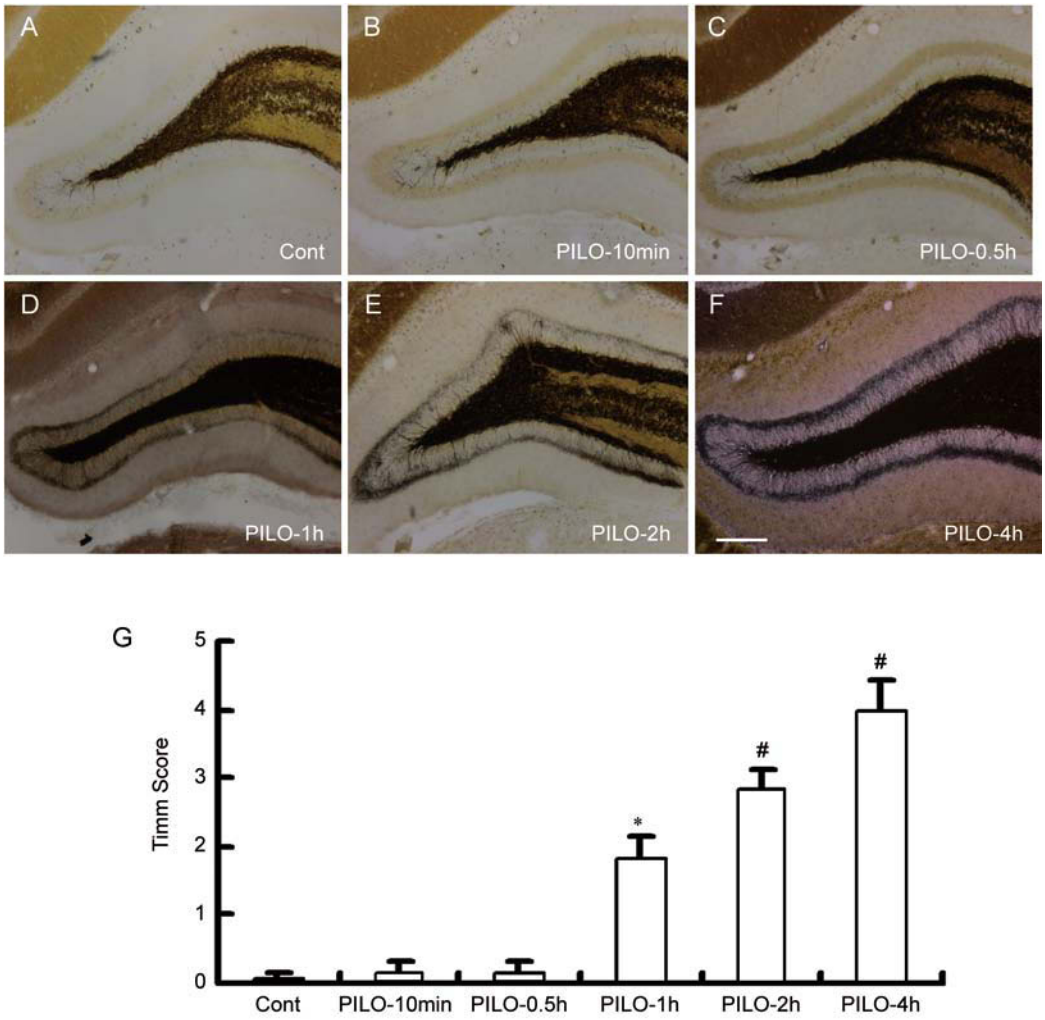

Fig. 3. Mossy fiber sprouting after different durations of SE. A–F: Representative sections with Timm staining showing mossy fiber sprouting in the inner molecular layer of the dentate gyrus from mice experienced different durations of SE. Mice were perfused 28 days after pilocarpine (PILO)-induced SE. Scale bar, 200 μm. G: Semi-quantitative analysis demonstrating a significant increase in Timm score with duration of SE. *P <0.01 compared with control (Cont) group, and #P <0.01 compared with 1-h SE group. One-way ANOVA (n = 6 mice/group).

number of hippocampal neurons were FJB-positive, located across the dentate hilus, CA3 and CA1 areas, especially in CA1 (Fig. 2F). However, in all other groups where mice experienced SE for <4 h, few hippocampal neurons were FJB-positive (Fig. 2A–E), even when the mice had developed spontaneous seizures (1-h and 2-h groups). Quantitative analysis of the death of hippocampal CA1 pyramidal cells confirmed that the number of dying neurons was significantly increased only in the 4-h SE group (Fig. 2G).

Mossy Fiber Sprouting Occurred in Mice after 1-h SE

Timm staining showed mossy fiber sprouting in mice that experienced >30-min SE (Fig. 3A–F). We further assessed the degree of sprouting using semi-quantitative analysis[10], and the results showed that the longer the SE duration, the stronger the Timm staining (Fig. 3G).

Correlation among SE Duration, Behavioral Changes and Histopathological Findings

A significant correlation was found between SE duration and subsequent spontaneous seizures (r = 0.986, P = 0.002), seizures and memory deficit (r = 0.941, P = 0.005), and seizures and Timm score (r = 0.979, P = 0.004). No significant correlation was found between spontaneous sei-

zures and numbers of FJB-positive neurons (r = 0.789, P = 0.113). These data, combined with those described above, demonstrate that 1-h SE is sufficient to develop seizures and impairment in spatial memory, and spontaneous seizures are associated with robust mossy fiber sprouting but not neuronal death.

DISCUSSION

Our main findings were: (1) pilocarpine reliably induced seizures and SE in a commonly-used strain of mice, and this led to subsequent spontaneous motor seizures (i.e., epilepsy), (2) one hour of SE was sufficient to develop epilepsy, and (3) the probability of developing epilepsy after SE depended on the duration of SE and was correlated with mossy fiber sprouting but not neuronal death.

Minimal SE Duration that Leads to the Development of Epilepsy and Its Clinical Implications

Through the activation of muscarinic receptors, pilocarpine excites neurons and reliably induces repeated convulsions (i.e., SE), which lead to subsequent epilepsy. In fact, pilocarpine-induced SE is one of the most commonly used animal models of acquired epilepsy[11]. However, regardless of its widespread application, the duration of SE used in this model is largely empirical ranging from 90 min to 3 h[5,6], and the minimal SE duration that leads to the development of epilepsy is unclear. This is an important question with close relevance to clinical patient care, and the answer to this question may provide a critical time-window for seizure intervention and epilepsy prediction and prevention. In this study, we found that SE lasting for 15–30 min was less likely to cause epilepsy later in life. However, SE lasting for 1 h was sufficient to develop epilepsy in ~25% of the mice (Table 2), and the probability of developing epilepsy and seizure frequency increased as SE duration was prolonged (Table 2). These data imply that the first hour of SE is the critical time-window, and it is important to intervene within the first hour to reduce the risk of developing epilepsy later.

Association of Post-SE Epilepsy with Mossy Fiber Sprouting *versus* Neuronal Death

A central hypothesis of the pathophysiological changes after SE is neuronal death and axonal sprouting, which ultimately lead to increased excitability of the neuronal network and the occurrence of spontaneous, recurrent seizures[2,12]. Perhaps the best-studied example of axonal sprouting during epileptogenesis is the mossy fiber sprouting phenomenon, where the dentate granule cells in the hippocampus in epileptic animals form aberrant new axons (also called mossy fibers) which project back to innervate the granule cells themselves[13-16]. Our study showed clear mossy fiber sprouting as indicated by Timm staining in mice after 1-h SE, and this became increasingly robust in animals with longer durations of SE (Fig. 3). Moreover, the degree of mossy fiber spouting paralleled the probability of spontaneous seizures (Table 2). Therefore, these data corroborate previous studies that axonal sprouting and synaptic organization are consistent pathophysiological changes during epileptogenesis[14-16].

Hippocampal neuronal loss (including the death of CA1 pyramidal cells) after SE has been repeatedly observed in previous studies[17,18]. However, repeated seizures cause neuronal injury and cell death depending on a number of variables such as the age of the animal, seizure type and duration[19]. For example, in the immature brain, recurrent seizures cause very little neuronal injury, and cell loss is not required for the development of epilepsy late in life[20-23]. Interestingly, in the present study we observed minimal neuronal death in the hippocampal CA1 area in mice that experienced SE for <4 h (Fig. 2), when robust mossy fiber sprouting was present and recurrent spontaneous seizures occurred (Table 2). We do not exclude some neuronal loss in the hilus region which is probably the main cause of mossy fiber sprouting, but the degeneration of the CA1 principal neurons was minimal, if any. These data suggest that while 1-h SE may not be sufficient to cause significant loss of hippocampal principal neurons, it is sufficient to lead to chronic epilepsy. Thus, it appears that in our mouse pilocarpine model, the development of epilepsy after a minimal duration of SE correlates more closely with mossy fiber sprouting than with neuronal death.

Several mechanisms might have contributed to the epileptogenesis without substantial cell death induced by 1-h SE. These mechanisms include changes in the upregulation of AMPA receptors[24], long-lasting changes in $GABA_A$ receptors[25], reduced HCN-currents[26], augmented endocannabinoid receptors[27], and activation of the mTOR signal pathway[8,9,28].

Different Susceptibility to Seizure-Related Outcome in Various Mouse Strains

Mouse strains differ in their susceptibility to drug-induced seizures, neuronal death, and behavioral changes. For example, C57BL6 mice are resistant to kainate seizure-induced neurodegeneration and mossy fiber sprouting, while ICR and FVB mice are vulnerable to neurotoxicity. However, in the pilocarpine-induced seizure model, C57BL6 mice exhibit clear neuronal death. They develop epilepsy with recurrent seizures as well and severe impairment of spatial learning and memory. In the present study, we used relatively cheap ICR mice and found that they exhibit neuronal death and mossy fiber sprouting as well as epilepsy development and memory deficits, suggesting that ICR mice are good candidates for multiple seizure-related experiments[29-31].

In summary, our data suggest that 1-h SE is sufficient to cause subsequent epilepsy in mice, and this is more closely associated with mossy fiber sprouting than neuronal death; and that the first hour during SE is the critical time-window for intervention in order to minimize the incidence of post-SE epilepsy.

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