Dual mechanisms of rapid expression of anxiety-related behavior in pilocarpine-treated epileptic mice

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Footnotes:

Abbreviations: BDNF, brain-derived neurotrophic factor; EEG, electroencephalogram; GFAP, glial fibrillary acidic protein; GS, generalized seizure; SE, status epilepticus.

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SUMMARY

A mouse model of epilepsy was generated by inducing status epilepticus (SE) for either 1.5 or 4.5 h with pilocarpine to study anxiety-related behaviors, changes in the electroencephalogram of the cerebral cortex and hippocampus, and expression of hippocampal proteins. The viability and rate of success of SE induction were high in C57BL/6N mice but not in C57BL/6J mice. C57BL/6N mice were immotile during the first 2 days after SE; however, by the third day, most mice were recovered and exhibited strong anxiety-related behaviors in response to the light/dark preference test and open field test. There was a striking difference in the temporal appearance of anxiety-related behavior between the two SE durations: 1.5 h SE mice exhibited strong anxiety-related behavior 3 days after SE that gradually attenuated over the next few weeks, whereas 4.5 h SE mice exhibited strong anxiety-related behavior 3 days after SE that persisted even at nearly 1 year after SE. Mice receiving both SE durations exhibited generalized seizures (GS) after SE; however, there was a marked difference in the timing and duration of GS appearance. Mice in the 4.5 h SE group exhibited spontaneous GS from 4 days to at least 96 days after SE. In contrast, mice in the 1.5 h SE group exhibited GS only within the first several days after SE; however, epileptic spike clusters continuously appeared in the cerebral cortex and hippocampus for up to twelve days after SE. Among the hippocampal proteins tested, only brain derived-neurotrophic factor (BDNF) exhibited altered expression in parallel with anxiety-related behavior. These results showed the possibility that BDNF expression in the hippocampus might cause anxiety-related behavior in adulthood.

KEYWORDS

Epilepsy; electroencephalogram; anxiety-like behavior; pilocarpine; BDNF; brain-derived neurotrophic factor

Introduction

Anxiety is a mental state that is evoked in anticipation of a potential or imminent threat; however, excessive or inappropriate anxiety can lead to pathological states. Anxiety is closely related to mental stress and depression, and the prevalence of anxiety disorder is increasing in modern society. Thus, the development of new anti-anxiety drugs and new methods of treatment are urgently required. The development of such treatments requires an understanding of the mechanisms underlying the expression of anxiety; however, little is known about the molecular and neuronal basis of anxiety (Gross and Hen, 2004).

Animal models have been successfully used to study the pathogenesis of many diseases. Animal models of acquired epilepsy have been developed by inducing status epilepticus (SE) via the administration of various drugs such as the chemo-convulsant drug pilocarpine (Mohajeri et al., 2004; Gröticke et al., 2007, 2008; Müller et al., 2009a,b; Hort et al., 1999; Detour et al., 2005; Dos Santos et al., 2005; de Oliveira et al., 2008; Scorza et al., 2009). Interestingly, these animal models exhibit typical anxiety-related behaviors as well as impaired cognitive functions, and thus, can be used to study the molecular and neuronal basis of anxiety. Many brain structural changes, including mossy fiber sprouting (Scharfman et al., 1999; Borges et al., 2003; Nadler 2007), reactive astrogliosis (Borges et al., 2003; Müller et al., 2009a,b; do Nascimento et al., 2012; Mazzuferi et al., 2012), and neuronal cell death in the CA1 region of the hippocampus (Gröticke et al., 2007; Müller et al., 2009a,b; Schauwecker, 2012), have been reported in these animal models. However, it remains unclear whether these changes contribute to the expression of anxiety-related behaviors, as the temporal appearance of anxiety-related behavior has not been investigated in previous studies. In the present study, therefore, we induced SE for either 1.5 or 4.5 h via pilocarpine to study anxiety-related behaviors, changes in the electroencephalogram (EEG) of the cerebral cortex and hippocampus, and expression of hippocampal proteins in SE-induced mice. We found that (i) anxiety-related behavior appeared 3 days after SE; (ii) anxiety-related behavior was either reversible or irreversible depending on the duration of SE induction; (iii) brain-derived neurotrophic factor (BDNF) levels changed in parallel with anxiety-related behavior; and (iv) despite an absence of spontaneous generalized seizures (GS), long-lasting changes in hippocampal EEG were observed in 1.5 h SE mice.

Materials and Methods

Animals

All procedures involving animals complied with the guidelines of the National Institutes of Health, and were approved by the Animal Experimentation and Ethics Committees of the Kitasato University School of Medicine. All efforts were made to minimize animal suffering and reduce the

number of animals used. C57BL/6N and C57BL/6J male mice (aged 8–9 weeks) were obtained from CLEA Japan (Tokyo, Japan) and housed under a 14 h:10 h light-dark cycle with lights on at 6:00 a.m. under constant temperature ($23 \pm 1^\circ\text{C}$) in a room with a clean air conditioning system and food and water provided *ad libitum*. We induced sustained seizures by administering pilocarpine, a muscarinic cholinergic agonist, using injection protocols derived from those described previously (Borges et al., 2003). To minimize the peripheral adverse effects of pilocarpine, we injected the mice with scopolamine methyl nitrate (2 mg/kg, i.p., in 0.9% NaCl) 30 min before pilocarpine injection (300 mg/kg, i.p., in 0.9% NaCl). Pilocarpine-uninjected control mice received scopolamine methyl nitrate but no pilocarpine. All drugs were obtained from Sigma (St. Louis, MO, USA). We classified seizures according to the criteria established by Racine (1972) and Schauwecker and Steward (1997) with slight modifications (Borges et al., 2003): normal activity (stage 0); rigid posture or immobility (stage 1); stiffened, extended, and often arched (Straub) tail (stage 2); partial body clonus, including forelimb or hindlimb clonus (seen rarely) or head bobbing (stage 3); whole-body continuous clonic seizures while retaining posture (stage 3.5); rearing (stage 4); severe whole-body continuous clonic seizures while retaining posture (stage 4.5); rearing and falling (stage 5); and tonic-clonic seizures with loss of posture or jumping (stage 6). SE was defined as sustained constant motor seizures of class 3.5 or higher, and was terminated by injecting 100–160 mg/kg phenobarbital after 1.5 h (1.5 h SE mice), and by injecting 30–40 mg/kg pentobarbital or 100–160 mg/kg phenobarbital after 4.5 h (4.5 h SE mice). After SE, all animals were administered 2 mL Lactec D (Otsuka Pharmaceutical Factory, Tokushima, Japan) subcutaneously and provided with soaked rodent food. Only pilocarpine-injected mice that developed SE for either 1.5 or 4.5 h were used for behavioral tests and electroencephalographic recordings.

Behavioral experiments

One week before the first behavioral experiment, mice were handled for 5 min once per day for 3 days. General activity was measured in an open field box placed in a sound-attenuated room. Mice were placed in the center of a brightly lit (650–700 lux) chamber of the apparatus ($50 \times 50 \times 30 \text{ cm}^3$). The movements of the animals were tracked with the LimeLight automatic monitoring system (Actimetrics Inc., Wilmette, IL, USA) for 30 min. The field was divided into 9×9 subareas, and the center area was defined as the inner 8×8 subareas. Horizontal motor activity (distance traveled) and central activity (distance traveled in the central area/total distance traveled) were evaluated.

The apparatus for the light/dark preference test consisted of two compartments of gray vinyl chloride plates and was placed in a sound-attenuated room. One compartment was a bright (250 lux) chamber ($25 \times 50 \times 40 \text{ cm}^3$) illuminated with a fluorescent lamp, and the other was a dark (0.5 lux) chamber ($25 \times 50 \times 40 \text{ cm}^3$). The two compartments were separated by a wall with a small opening ($8 \times 16 \text{ cm}^2$). Two pairs of 24×24 -array infrared photosensors were attached to the outer wall, equally spaced in rows 2.5 and 6.5 cm from the floor. The lower row of photocells was used to detect the

location of the mouse, and the upper row was used to detect rearing behavior. The sensor state was sampled every 0.1 s. A mouse was placed in the corner of the light chamber distal to the opening, and its behavior was recorded via computer for 30 min. The mouse was considered to have entered a new area when the center of the body was in that area. The apparatus was cleaned between sessions with water.

Surgery

Mice were implanted with EEG and electromyogram electrodes under sevoflurane inhalation anesthesia (3–4% sevoflurane/oxygen flow 0.5 L/min) and subcutaneously injected xylocaine. Bipolar electrodes (two stainless-steel wires, 0.2 mm in diameter, tip separation of 0.5 mm) were implanted in either the left dorsal hippocampus [posterior (P): 2.0 mm from the bregma, lateral (L): 1.4 mm from the midline]. Three stainless-steel miniature screws (Φ 1.0 mm), which served as epidural EEG electrodes, were placed over the left frontal cortex [anterior (A): 1.2, L: 1.0], right anterior region (reference electrode; A: 2.5, L: 0.6), and right occipital region (ground electrode; P: 2.5, L: 2.0). EEG recordings were started on the day of SE induction.

EEG recordings

Hippocampal EEG was recorded between the long and short bipolar electrodes, and cortical EEG was recorded between the anterior and frontal cortical screw electrodes. The signal from the recording electrodes was amplified and filtered (time constant: 0.01 s, high cut: 30 Hz). In addition, a 50-Hz notch filter was applied. The amplified signal was digitized at 500 Hz with a data translation A/D board and was recorded directly onto a PC hard drive by using Multi Scope 2100 (Medical Try System, Tokyo Japan). Simultaneously, all mice were continuously video monitored using two charge-coupled device (CCD) cameras set in the diagonal direction during the experimental periods. The cameras were capable of infrared recording during the dark phase. Mice were housed with free access to food and water in clear plastic cages (one per cage) to allow optimal video observation in a sound-attenuated box. During the experimental period, mice were maintained in a 12 h:12 h light-dark cycle with lights on at 8:00 a.m. The EEG recording was interrupted from 09:00 to 10:00 a.m. each day for cage cleaning and feeding.

Analysis of EEG data

All EEG data were analyzed with Multi Scope 2100 software. Because the absolute value of the EEG data varied among different mice and different experimental days, possibly owing to different electrode conditions, a standard value for normalization was determined for each mouse on each experimental day. The average EEG values during 5-min intervals in which the mouse was

awake were measured at five different time points each day, and the mean of these values was used as the standard value of the mouse on that day. For spike counting, the number of spikes in which the amplitude was 10 times larger than the standard value was counted. Seizures were identified by EEG and video monitoring.

Antibodies

We used rabbit anti-gial fibrillary acidic protein (GFAP; Sigma, G3893), rabbit anti-actin (BD Biosciences, San Jose, CA, USA, 612657), rabbit anti-GluN1 (Cell Signaling Technology, Danvers, MA, USA, #5704), anti-GluN2A (Millipore, Billerica, MA, USA, 07-632), rabbit anti-GluN2B (Cell Signaling Technology, #4212), anti-GluA3 (Cell Signaling Technology, #4676), and anti-PSD-95 (Cell Signaling Technology, #3409 and #3450). A mouse monoclonal anti-VAMP-2 antibody (3D10) was generously provided by Dr. Shunji Kozaki of Osaka Prefecture University. Mouse monoclonal anti-BDNF antibody (5D7) and anti GluA2 antibody (7G6) were obtained by immunizing mice with synthetic peptides TMDSKKRIGWRFIR (residues 221–234 of rat pro-BDNF) and YKEGYNVYGIESVKI (residues 869–883 of mouse GluA2), respectively. Anti-peptide antibodies were obtained by immunizing rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin as previously described (Iwasaki et al., 2000). The amino acid sequences of the synthetic peptides were as follows: anti-SNAP23, KNRIDIANTRAKKLIDS (residues 194–210 of rat SNAP-23); anti-GluA1, QSINEAIRTSTLPRNSG (residues 848–864 of mouse GluA1); anti-Cacng2, DSKDSLHANTANRRITTPV (residues 306–323 of rat Cacng2); anti-Cacng8, SAGFLTLHNAFPKEA (residues 358–372 of mouse Cacng8); anti-VGluT1, KRQEGAETLELSADGRPV (residues 25–42 of mouse VGluT1); and anti-VGAT, GDEGAEAPVEGDIHYQR (residues 71–87 of mouse VGAT). All of the antibodies were affinity-purified from the antiserum using an antigen peptide-conjugated Sepharose 6B column (Sigma, St. Louis, USA).

Western blotting

For western blotting, the hippocampus was removed and homogenized in 0.5 mL sodium dodecyl sulfate (SDS) sample buffer [70 mM SDS, 3.3% (w/v) glycerol, 0.03 mM bromophenol blue, 125 mM Tris-HCl, pH 6.8] with an ultrasonicator on ice. The homogenates were boiled for 10 min and stored as 1 mg/mL frozen stocks. Dithiothreitol (DTT) (20 mM) was added and the samples were boiled for 3 min before use. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on gradient polyacrylamide gels (e-PAGEL, ATTO, Tokyo, Japan) in the presence of 50 mM DTT. Proteins were electrophoretically transferred onto polyvinylidene fluoride membranes (Immobilon-P, Merck Millipore, Darmstadt, Germany) with a semidry transblotting apparatus. The membranes were blocked with 5% (w/v) nonfat milk in a mixture of Tris-Buffered Saline and Tween 20 (TBST; 0.05% Tween 20, 150 mM NaCl, 25 mM Tris-HCl, and pH 7.5) and incubated with various antibodies

overnight at 4 °C. After washing with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies in TBST containing 1% nonfat milk. After washing with TBST, immunoreactive bands were visualized with Super Signal chemiluminescence (Thermo Fisher Scientific, Waltham, USA) in a linear range using a luminescence image analyzer with electronically cooled CCD camera systems (LAS-4000, Fujifilm, Tokyo, Japan). ImageQuant TL (Fuji Photo Film Co.) was used for image quantification of the bands.

Statistics

Data are presented as the mean $\pm$ standard error of the mean (SEM). Data from two groups were compared by two-tailed unpaired Student's *t*-test. Data from three or more groups were analyzed by one-way or two-way analysis of variance (ANOVA) tests followed by Tukey-Kramer's honest significant difference *post hoc* tests. A *p* value <0.05 was considered statistically significant.

Results

Strain-dependent difference in the viability and SE induction of the pilocarpine mouse

The viability of the pilocarpine mouse model depends on the strain and the animal suppliers (Borges, 2003; Curia et al., 2008; Schauwecker, 2012). We injected pilocarpine (100–300 mg/kg) into C57BL/6J mice with or without other drugs, including pentylenetetrazol (10–50 mg/kg) and lithium chloride (125 mg/kg); none of the mice in which SE was established were alive within 1 day after pilocarpine treatment. In a striking contrast, the viability and rate of success of SE induction was high in C57BL/6N mice: 85.3% (307 of 360) of mice survived pilocarpine injection, and 66.1% (238 of 307) of the surviving mice exhibited SE for 4.5 h. Thus, we used the C57BL/6N mouse for all remaining experiments.

Rapid expression of anxiety-related behavior after SE in the pilocarpine mouse

The development of spontaneous recurrent seizure depends on the duration of SE (Klitgaard et al., 2002; Gröticke et al., 2007; Curia et al., 2008); thus, we tested both 1.5 and 4.5 h SE durations. The mice were immotile during the first 2 days after SE, making the assessment of behavior difficult. However, by the third day, most mice had recovered and showed a strong preference for the dark room during the light/dark preference test. Figure 1 shows the total distance (A and D), average velocity (E), percentage of photobeam crossings in the light room (B and F), and typical traces (C) from the light/dark preference test in control, 1.5 h SE, and 4.5 h SE mice at 3 days after SE. The quantity of locomotor activity was close to normal in most 1.5 h SE mice such that the total distance exceeded 50% of that observed in control mice in 12 of 13 1.5 h SE mice (Fig. 1A); however, in one

mouse (mouse no. 1), total distance was only 11% of control mice. Less recovery was observed in 4.5 h SE mice such that the total distance was less than 11% of that in control mice in 3 of 14 4.5 h SE mice (Fig. 1A). We eliminated these four mice (one 1.5 h SE mouse and three 4.5 h SE mice) from subsequent analysis, because these mice did not move from the start position in the light room (Fig. 1B). There was no significant difference in the total distance (Fig. 1D) and average velocity (Fig. 1E) between control mice and 1.5 h SE mice. In contrast, the total distance ($p < 0.0001$, Fig. 1D) and average velocity ($p = 0.0004$, Fig. 1E) were significantly lower in 4.5 h SE mice than in control mice. Interestingly, the percentage of photobeam crossings in the light room was significantly lower ($p < 0.00001$) in both 1.5 h SE mice ($9.66 \pm 15.56\%$, mean $\pm$ standard deviation, $n = 12$ mice) and 4.5 h SE mice ($3.60 \pm 5.33\%$, $n = 11$ mice) compared to control mice ($39.8 \pm 8.9\%$, $n = 8$ mice) (Fig. 1F). These results indicate that anxiety-related behavior appears within 3 days after SE in both 1.5 h SE and 4.5 h SE mice.

Transient and stable expression of anxiety-related behavior in the pilocarpine mouse

There was a striking difference in the temporal appearance of anxiety-related behavior between 1.5 h SE mice and 4.5 h SE mice. Figure 2 shows the time-dependent changes in the percentage of photobeam crossings in the light room in control mice (A), 1.5 h SE mice (B), and 4.5 h SE mice (C). Typical traces are superimposed on each graph. There was no significant time-dependent change in the percentage of photobeam crossings in the light room in control mice across 30 days (Fig. 2A). Mice in the 1.5 h SE group showed strong avoidance of the light room at 3 days after SE; however, the percentage of photobeam crossings in the light room gradually increased over the following days (Fig. 2B). The average percentage of photobeam crossings in the light room was markedly decreased at 3–4 days after SE ($3.45 \pm 0.92\%$, $n = 14$ mice, $p < 0.0001$) compared with control mice ($46.8 \pm 1.8\%$, $n = 19$ mice), but increased to $23.1 \pm 1.8\%$ ($n = 19$ mice) and $32.4 \pm 7.6\%$ ($n = 20$ mice) at 6–11 and 13–22 days after SE, respectively (Fig. 3A). Full recovery was not observed, as recovery was significantly lower (69.4% of the control, $p < 0.0001$) compared with control mice at 13–22 days after SE. In a striking contrast, the average percentage of photobeam crossings in the light room markedly decreased ($5.39 \pm 1.09\%$, $n = 29$ mice, $p < 0.0001$ compared with control mice) at 3–10 days after SE for 4.5 h SE mice and remained decreased at 14–21 days after SE ($3.09 \pm 0.87\%$, $n = 28$ mice) (Fig. 3A). There were no significant changes in the amount of time in motion among the control, 1.5 h SE, and 4.5 h SE mice (Fig. 3B). The average velocity tended to increase slightly but significantly after SE in both the 1.5 h SE and 4.5 h SE mice. Average velocity was 17.11 ± 0.22 cm/s ($n = 19$) in control mice compared with 19.62 ± 0.24 cm/s ($n = 19$, $p = 0.0015$) and 19.88 ± 0.32 cm/s ($n = 20$, $p = 0.0003$) in 1.5 h SE mice at 6–11 days and 13–22 days after SE, respectively, and 18.19 ± 0.56 cm/s ($n = 29$, $p = 0.41$) and 19.97 ± 0.22 cm/s ($n = 28$, $p < 0.0001$) in 4.5 h SE mice at 3–10 days and 14–21 days after SE, respectively (Fig. 3C). Thus, the total distance also increased significantly in both the 1.5 h SE and 4.5 h SE mice (Fig. 3D). The number of rearings per minute in the light

room was lower in both the 1.5 h SE mice (1.37 ± 0.52 times/min, $n = 13$) and the 4.5 h SE mice (3.53 ± 0.62 times/min, $n = 26$) compared with control mice (11.2 ± 0.80 times/min, $n = 19$) at 3–4 days and 3–10 days after SE, respectively (Fig. 3E). The number of rearings in 1.5 h mice exceeded that in control mice at 6–11 days after SE; however, no such recovery was observed in the 4.5 h SE mice (Fig. 3E). There were no significant differences between control and 4.5 h SE mice in the number of rearings per minute in the dark room; however, the number of rearings was significantly higher in the 1.5 h SE mice [13.5 ± 0.65 times/min ($n = 19$, $p < 0.0001$) and 12.5 ± 0.52 times/min ($n = 20$, $p = 0.0005$) at 6–11 and 13–22 days after SE, respectively] compared with control mice (7.90 ± 0.64 times/min, $n = 19$) (Fig. 3F).

Appearance of anxiety-related behavior in the open field test

A transient expression of anxiety-related behavior also was observed in the open field test. Figure 4A shows typical traces in control mice (top three panels), 1.5 h SE mice (middle three panels), and 4.5 h SE mice (bottom three panels). Thigmotaxis, a typical anxiety-related behavior in the open field test, was observed in 1.5 h SE mice at 2 days after SE, and in 4.5 h SE mice on all days tested after SE. Figures 4B and 4C show the total distance and percentage of distance traveled in the central area for control, 1.5 h SE, and 4.5 h SE mice. There was no significant change in total distance among control mice (79.7 ± 4.2 m/30 min, $n = 10$), 1.5 h SE mice (71.8 ± 24.3 m/30 min, $n = 4$, $p = 0.998$ compared with control), and 4.5 h SE mice (72.0 ± 11.0 m/30 min, $n = 5$, $p = 0.997$ compared with control) within 6 days after SE. However, total distance increased markedly thereafter for both 1.5 h SE mice (158.2 ± 9.9 m/30 min, $n = 7$, $p = 0.0057$ compared with control) and 4.5 h SE mice (132.4 ± 14.2 m/30 min, $n = 17$, $p = 0.03$ compared with control) within 19 days after SE. Mice in the 1.5 h SE group exhibited transient thigmotaxis within 6 days after SE, and the percentage of distance traveled in the central area was significantly lower ($17.8 \pm 3.2\%$, $n = 4$, $p = 0.0006$ compared with control) than that of control mice ($35.5 \pm 1.9\%$, $n = 10$), but increased ($40.5 \pm 4.8\%$, $n = 7$, $p = 0.54$ compared with control) within 19 days after SE. In a striking contrast, 4.5 h SE mice exhibited stable thigmotaxis, and the percentage of distance traveled in the central area was significantly lower than that of control mice both within 6 days ($4.5 \pm 0.88\%$, $n = 5$, $p < 0.0001$ compared with control) and 19 days ($4.1 \pm 1.00\%$, $n = 17$, $p < 0.0001$ compared with control) after SE.

Electroencephalograph of 1.5 h SE and 4.5 h SE mice

Both 1.5 h SE mice and 4.5 h SE mice exhibited GS in the period after SE; however, there was a marked difference in the time of appearance of GS. As shown in Fig. 5A, 1.5 h SE mice exhibited GS only within several days after SE. The number of GS per day was highest at 2 days after SE and then decreased gradually; no spontaneous GS was observed from 7 days after SE. In contrast, there was a GS-free latent period for approximately 3 days in 4.5 h SE mice. Mice in the 4.5

h SE group exhibited spontaneous GS from 4 days after SE; however, the number of spontaneous GS each day varied in a cyclic manner (Fig. 5A), as previously reported (Goffin et al., 2007; Mazzuferi et al., 2012). There were no noticeable differences in the GS waveform between 1.5 h SE mice and 4.5 h SE mice (Fig. 5B); however, the incidence of GS with longer durations increased in 4.5 h SE mice compared with 1.5 h SE mice (Fig. 5C), and the average seizure duration was significantly ($p < 0.0001$) longer in 4.5 h SE mice (38.1 ± 1.24 s, $n = 109$) than in 1.5 h SE mice (26.8 ± 1.82 s, $n = 43$) (Fig. 5D).

Although 1.5 h SE mice did not express spontaneous GS from 7 days after SE, there were striking changes in the EEGs of the cerebral cortex and hippocampus. The spike number increased in both the cerebral cortex and hippocampus at 5 days after SE in 1.5 h SE mice (Figs. 6A and 6B). Many spikes appeared in clusters that continued for >60 min. The number of minutes per day exhibiting >10 spikes/min was higher in the cerebral cortex (254.1 ± 44.9 min, $n = 4$) and hippocampus (249.2 ± 79.0 min, $n = 4$) in 1.5 h SE mice at 4 days after SE than in control mice (cerebral cortex, 4.0 ± 2.1 min, $n = 8$; hippocampus, 62.2 ± 20.4 min, $n = 8$). Spike clusters appeared during both the light and dark phases of the 24-hour day (Fig. 6B). The total spike cluster duration per day decreased remarkably in the cerebral cortex, but remained high in the hippocampus at 12 days after SE (Fig. 6C). However, although many spike clusters appeared in the cerebral cortex at 4 days after SE in 4.5 h SE mice, few clusters appeared at 12 days after SE (Figs. 6B and 6C).

Changes in structure and protein expression level in the hippocampus after SE

As previously reported, 4.5 h SE mice exhibited various changes in hippocampal structures, including the appearance of reactive astrocytes, mossy fiber sprouting in the dentate gyrus, and neuronal cell loss in the CA1 and CA3 regions (data not shown). In a striking contrast, mossy fiber sprouting and neuronal cell loss in the CA1 and CA3 regions were not observed in 1.5 h SE mice even at 14 days after SE. We conducted quantitative immunoblotting of hippocampal proteins from 1.5 h SE mice and 4.5 h SE mice prepared at 4 and 18 days after SE. There were no significant changes in the amount of actin and SNAP-23, a ubiquitous homologue of SNAP-25, in both 1.5 h SE and 4.5 h SE mice at 4 and 18 days after SE (Figs. 7A and B). In contrast, the protein level of GFAP increased remarkably at 4 days after SE, a level that was maintained at 18 days after SE in both 1.5 h SE and 4.5 h SE mice (Fig. 7C). Interestingly, the expression of BDNF increased transiently at 4 days after SE (3.82 ± 0.59 times as large as the control level, $n = 6$, $p < 0.0001$) and returned to the control level (1.65 ± 0.16 times as large as the control level, $n = 6$) at 18 days after SE in 1.5 h SE mice. In a striking contrast, BDNF increased at 4 days after SE (4.71 ± 0.52 times as large as the control level, $n = 6$), but was retained at a similar level at 14 days after SE in 4.5 h SE mice (6.13 ± 0.59 times as large as the control level, $n = 6$). There was a strong negative correlation ($r = -0.75$) between the amount of BDNF and the percentage of photobeam crossings in the light room, a quantitative marker of anxiety-related behavior in the light/dark preference test (Fig. 7E).

Although there were some differences among subtypes, the amounts of glutamate receptors, including GluN1, GluN2A, GluN2B, GluA1, GluA2, and GluA3, and their scaffold proteins, including Cacng2, Cacng8, and PSD-95, decreased at 4 days after SE in 1.5 h SE and 4.5 h SE mice with no recovery at 18 days after SE (Fig. 8). The amount of VAMP-2, a presynaptic protein, also decreased at 4 days after SE, and no recovery was observed in either 1.5 h SE or 4.5 h SE mice at 18 days after SE. The amount of VGluT, a presynaptic protein of excitatory neurons, decreased significantly in 4.5 h SE mice at 4 and 18 days after SE, whereas no significant decrement was observed even at 18 days after SE in 1.5 h SE mice. In a striking contrast, a significant decrease was observed in the amount of VGAT, a presynaptic protein of inhibitory neurons, in both 1.5 h SE and 4.5 h SE mice at 4 and 18 days after SE.

Discussion

Because epileptogenesis depends on the SE duration in the pilocarpine model (Curia et al., 2008), we induced SE for both 1.5 and 4.5 h in the present study. The viability of the pilocarpine mouse model depended on both the mouse strain and animal supplier (Borges et al., 2003; Curia et al., 2008; Schauwecker, 2012). We observed reasonable viability with C57BL/6N but not with C57B/6J mice purchased from CLEA Japan.

Differences in epileptogenesis depends on the duration of SE induction

As reported previously (Goffin et al., 2007; Scorza et al., 2009; Mazzuferi et al., 2012), 4.5 h SE mice exhibited spontaneous GS from 4 days to at least 96 days after SE. The existence of a latent period before the appearance of spontaneous epileptic seizures remains controversial (Raol et al., 2006; Goffin et al., 2007; Bumanglag and Sloviter, 2008; Dudek and Staley, 2011a,b). However, in the present study, continuous long-term EEG monitoring clearly revealed the existence of a latent period in the pilocarpine model. In a striking contrast, 1.5 h SE mice exhibited spontaneous GS on the days immediately following SE with a peak at 2 days after SE; however, no spontaneous GS was observed from 7 days after SE. It is unlikely that GS observed on the days immediately following SE were due to residual pilocarpine, as no GS were observed during the same time period in 4.5 h SE mice.

Although 1.5 h SE mice were not epileptic based on the absence of spontaneous GS (except for the period immediately following SE), we observed long-term hyperactivity marked by clusters of epileptic spikes in the cerebral cortex and hippocampus. Recently, it was reported that rats without SE exhibited spontaneous GS 8 months after SE induction (Navarro Mora et al., 2009). Thus, the long-lasting hippocampal hyperactivity observed in 1.5 h SE mice may have possibly contributed to the induction of GS after a long latent period. The results of immunoblotting analysis of hippocampal proteins revealed no significant difference in the amount of VGluT1, a marker protein of excitatory neurons between 1.5 h SE mice and control mice; however, there was a significant reduction of VGAT,

a marker protein of inhibitory neurons, without recovery even at 18 days after SE. Thus, the partial loss of inhibitory neurons may be responsible for the hyperactivity observed in the hippocampus and cerebral cortex of 1.5 h SE mice.

Differences in anxiety-related behavior depends on the duration of SE induction

Many reports have shown that pilocarpine rats and mice exhibit many cognitive disorders and anxiety-related behaviors after epileptogenesis; however, the brain changes that underlie these abnormal behaviors has remained unclear because behavior analysis was not conducted until several weeks after SE (Mohajeri et al., 2004; Detour et al., 2005; Dos Santos et al., 2005; Grötcke et al., 2007, 2008; Müller et al., 2009a,b). To clarify these problems, we conducted behavior tests as early as possible after SE induction. It was not possible to conduct behavior tests on the 2 days immediately following SE because the mice were immotile. At 3 days after SE, 12 of 13 mice in the 1.5 h SE group and 11 of 13 mice in the 4.5 h SE mice moved more than 50% and 30% of the total distance of control mice, respectively. Among 1.5 h SE mice, 10 of 12 of the recovered mice exhibited anxiety-related behavior such that the percentage of crossing in the light room was <7.2%. The remaining 2 mice did not exhibit significant anxiety-related behavior such that the percentage of crossings in the light room was 50.5% and 32.8%, respectively. In contrast, all 11 of the recovered 4.5 h SE mice exhibited strong anxiety-related behavior, and the average percentage of crossings in the light room was $3.60 \pm 5.33\%$. These results clearly demonstrate that anxiety-related behavior was induced by changes in the brain that occurred by 3–4 days after SE.

In the present study, we found a striking difference in the nature of the appearance of anxiety-related behaviors between 1.5 h SE and 4.5 h SE mice such that anxiety-related behavior was transient in 1.5 h SE mice and long-lasting in 4.5 h SE mice. Despite this difference, there were several common features in the anxiety-related behavior of these mice. The total moving time and the average velocity were not changed in either group. The total number of rearings in both the light room and the dark room tended to decrease in both 1.5 h SE and 4.5 h SE mice. Transient and long-lasting anxiety-related behaviors were also observed in the open field test in 1.5 h SE mice and 4.5 h SE mice, respectively. These results suggest that there are some common mechanisms in the appearance of anxiety-related behaviors in 1.5 h SE and 4.5 h SE mice.

Structural changes in the brain observed in the pilocarpine mouse

As reported previously (Grötcke et al., 2007; Müller et al., 2009a,b; Schauwecker, 2012), neuronal cell loss of CA1 pyramidal neurons occurred by 3–4 days after SE in 4.5 h SE mice. This neuronal cell loss was accompanied by a concomitant reduction of various synaptic proteins, including several subunits and scaffold proteins of glutamate receptors as well as synaptic vesicular proteins such as VAMP-2, VGluT, and VGAT. Thus, although the possibility exists that a loss of CA1

pyramidal neurons may be involved in the expression of anxiety-related behavior in 4.5 h SE mice, further studies are needed. Marked mossy fiber sprouting of dentate granule cells has been observed in pilocarpine mice and rats, and neuropeptide Y-positive immunoreactivity appeared outside of the granular layer of the dentate gyrus (Scharfman et al., 1999; Borges et al., 2003; Nadler, 2007). In the present study, we observed mossy fiber sprouting in 4.5 h SE mice only at approximately 4 weeks after SE; thus, mossy fiber sprouting does not seem to be involved in the expression of anxiety-related behavior, which appeared 3–4 days after SE.

Anxiety-related behavior appeared without CA1 neuronal cell loss in 1.5 h SE mice, which suggests that neuronal cell loss is not necessary for the transient expression of anxiety-related behavior in 1.5 h SE mice. Although the amount of various synaptic proteins, including the NR2 subunit of the *N*-methyl-D-aspartate (NMDA) receptor and various subunits of the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, decreased significantly by 4 days after SE in 1.5 h SE mice, these reductions did not seem to be involved in the expression of anxiety-related behavior, as such behavior was decreased by 2 weeks after SE without any recovery of these proteins.

Possible involvement of BDNF expression in the appearance of anxiety-related behavior

The expression of BDNF increases markedly after an epileptic seizure (Binder et al., 2001). In the present study, we found a strong negative correlation between the amount of BDNF in the hippocampus and the extent of anxiety-related behavior in the light/dark preference test. Although several studies have suggested a relationship between the expression level of BDNF and anxiety-related behavior, findings have been inconsistent across studies (Domingos da Silveira da Luz et al., 2013; Autry and Monteggia, 2012). Anxiety was increased in an elevated plus-maze test and in the open field test in BDNF transgenic mice (Govindarajan et al., 2006). Similarly, female BDNF transgenic mice exhibited anxiety-related behavior in the light/dark preference test with a concomitant increase in BDNF expression in the cerebral cortex and hippocampus (Papaleo et al., 2011); however, anxiety-related behavior did not appear in BDNF transgenic mice of other strains (Croll et al., 1999). A sex difference also was observed in frontal cortex-specific BDNF knockout mouse such that anxiety-related behavior decreased in the elevated plus-maze test and open field test in female mice only (Monteggia et al., 2007). In humans, anxiety increased in those with a genetic background, resulting in the elevation of activity-dependent BDNF release (Lang et al., 2005). In contrast, heterozygous BDNF knockout mice exhibited anxiety-related behavior in the open field test and elevated plus-maze test (Chen et al., 2006). A BDNF mutant mouse having a single amino acid substitution of Val66Met, which decreases activity-dependent BDNF release without changing the BDNF expression level, also exhibited anxiety-related behavior in the open field test and elevated plus-maze test (Chen et al., 2006). The expression level of BDNF decreased in the frontal cortex, hippocampus, hypothalamus, and midbrain in C57BL/6J mice, which were kept isolated and

exhibited anxiety-related behavior in the open field test and elevated plus-maze test (Berry et al., 2012). The expression of BDNF increased by the application of a serotonin reuptake inhibitor (Nibuya et al., 1995), which decreased anxiety in these mice (Dulawa et al., 2004). Together these results suggest that anxiety-related behaviors arise through either the reduction of BDNF expression or the reduction of activity-dependent BDNF release.

As environmental stress during the neonatal period affects anxiety behaviors in adulthood, results in BDNF knockout mice, in which the expression and function of BDNF are altered beginning in the postnatal period, should be interpreted carefully. The present study clearly shows that changes in BDNF expression in adulthood may affect the expression of anxiety-related behavior; however, further studies are needed to clarify the role of BDNF in anxiety-related behavior.

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Figure legends

Fig. 1 Rapid appearance of anxiety-related behavior in mice in the light/ dark preference test after status epilepticus (SE). The light/dark preference test was conducted at 3 days after SE in pilocarpine-treated mice in whom SE was induced for either 1.5 or 4.5 h. (A) Total distance over 15 min for 1.5 h SE mice (*left*) and 4.5 h SE mice (*right*). (B) Percentage of photobeam crossings in the light room for 1.5 h SE mice (*left*) and 4.5 h SE mice (*right*). The results of 1.5 h SE mice (n = 13) and 4.5 h SE mice (n = 14) are shown with orange and red bars, respectively; the mean $\pm$ SEM of pilocarpine-uninjected control mice (n = 8) is shown with green bars. (C) Typical traces of control mice (Cont., *top*), 1.5 h SE mice (*middle*), and 4.5 h SE mice (*bottom*). The left and right halves are the light and dark room, respectively. The mouse number indicated at the bottom correlates with the number shown in A and B. (D) The total distance, (E) average velocity, and (F) percentage of photobeam crossings in the light room for control mice (green bars, n = 8), 1.5 h SE mice (orange bars, n = 12, nos. 2–13), and 4.5 h SE mice (red bars, n = 11, nos. 4–14). Statistical analysis was performed with one-way analysis of variance followed by Tukey-Kramer's honest significant difference *post-hoc* tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control mice.

Fig. 2 Time-dependent changes in anxiety-related behavior in the light/dark preference test after status epilepticus (SE). The percentage of photobeam crossings in the light room was measured in pilocarpine-uninjected control mice (A), pilocarpine-injected 1.5 h SE mice (B), and pilocarpine-injected 4.5 h SE mice (C) on various days. The dashed line indicates the average value of control mice. Typical traces are shown on the top of each graph, and the days of measurement were as follows: (A) 4, 7, 11, 21, and 29 days after scopolamine injection; (B) 3, 5, 11, 14, and 22 days after SE; and (C) 3, 6, 10, 14, and 307 days after SE (from *left* to *right*).

Fig. 3 Time-dependent changes of the various parameters in the light/dark preference test. (A) Percentage of photobeam crossings in the light room; (B) total moving time; (C) average velocity; (D) total distance; (E) rearing number per minute in the light room; and (F) rearing number per minute in the dark room. The measurements shown in Fig. 2 were grouped as follows by the period after status epilepticus (SE): control mice (green bar), 4–22 days (n = 19); 1.5 h SE mice (orange bars), 3–4 days (n = 14), 6–11 days (n = 19), and 13–22 days (n = 20); 4.5 h SE mice (red bars), 3–10 days (n = 29) and 14–21 days (n = 28). Statistical analysis was performed with one-way analysis of variance followed by Tukey-Kramer's honest significant difference *post-hoc* tests. * $p < 0.05$, ** $p < 0.01$ compared with the control.

Fig. 4 Time-dependent changes in anxiety-related behavior in the open field test after status epilepticus (SE). (A) Typical traces for control mice (*top three*), 1.5 h SE mice (*middle three*), and 4.5 h SE mice (*bottom three*). The days of measurement were as follows: control mice, 6, 19, and 28 days

after SE; 1.5 h SE mice, 2, 6, and 9 days after SE; 4.5 h SE mice, 8, 16, and 58 days after SE (from *left to right*). (B) Total moving distance. (C) Percentage in the central area. The measurements were grouped as follows by the period after SE: control mice (green bar), 6–19 days ($n = 10$); 1.5 h SE mice (orange bars), 2–6 days ($n = 4$) and 9–16 days ($n = 7$); and 4.5 h SE mice (red bars), 6 days ($n = 5$) and 7–19 days ($n = 17$). Statistical analysis was performed with one-way ANOVA followed by Tukey-Kramer's honest significant difference *post-hoc* tests. $*p < 0.05$, $**p < 0.01$ compared with the control.

Fig. 5 Generalized seizures (GS) in 1.5 h status epilepticus (SE) and 4.5 h SE mice after SE. (A) Average number of spontaneous GS at various days after SE for 1.5 h SE mice (orange triangles, $n = 7$) and 4.5 h SE mice (red circles, $n = 6$). (B) Typical electroencephalographic waveforms of the cerebral cortex (Cor, red) and hippocampus (Hip, blue) in GS for 1.5 h SE mice (*top*) and 4.5 h SE mice (*bottom*). (C) Distribution of GS duration for 1.5 h SE mice (orange bar, $n = 43$) and 4.5 h SE mice (red bar, $n = 109$). (D) Average duration of GS for 1.5 h SE mice (orange bars, $n = 43$) and 4.5 h SE mice (red bars, $n = 109$). Statistical analysis was performed with Student's *t*-test. $**p < 0.01$.

Fig. 6 Epileptic spike clusters in 1.5 h status epilepticus (SE) mice and 4.5 h SE mice. (A) Typical electroencephalographic (EEG) records from the cerebral cortex (Cor, red) and hippocampus (Hip, blue) of control mice (Cont), 1.5 h SE mice (1.5 h SE), and 4.5 h SE mice (4.5 h SE) measured at 5 and 12 days after SE. Expanded EEGs are shown on the right. (B) The number of spikes per minute is shown for the cerebral cortex (*left*, red) and hippocampus (*right*, blue) of control mice (a and f), 1.5 h SE mice at 5 days (b and g) and 12 days after SE (c and h), and 4.5 h SE mice at 5 days (d and i) and 12 days after SE (e and j). Green arrows show the time of EEG recordings shown in A. (C) Total duration of spike clusters per day in the cerebral cortex (*left*, red) and hippocampus (*right*, blue) for control mice and 1.5 h SE mice (*top*) and control mice and 4.5 h SE mice (*bottom*) at 5 and 12 days after SE. Statistical analysis was performed with one-way analysis of variance followed by Tukey-Kramer's honest significant difference *post-hoc* tests. $*p < 0.05$, $**p < 0.01$ compared with the control.

Fig. 7. Expression levels of actin (A), SNAP-23 (B), glial fibrillary acidic protein (GFAP) (C), and brain-derived neurotrophic factor (BDNF) (D) in the hippocampus of control mice (green bar), 1.5 h status epilepticus (SE) mice (orange bars), and 4.5 h SE mice (red bars). The samples were prepared at 4 and 18 days after SE as indicated. The hippocampal proteins were separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein amounts were determined by quantitative immunoblotting. The values are represented relative to those of control mice. (E) Correlation between BDNF expression level and percentage of photobeam crossings in the light area in the light/dark preference test. Statistical analysis was performed with one-way analysis of variance followed by Tukey-Kramer's honest significant difference *post-hoc* tests. $*p < 0.05$, $**p <$

0.01 compared with the control.

Fig. 8. Expression levels of *N*-methyl-D-aspartate receptors (GluN1, GluN2A, and GluN2B), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (GluA1, GluA2, and GluA3), scaffold proteins (Cacng2, Cacng8, and PSD-95), and presynaptic proteins (VAMP-2, VGluT1, and VGAT) in the hippocampus of control mice (green bar), 1.5 h status epilepticus (SE) mice (orange bars), and 4.5 h SE mice (red bars). Samples were prepared at 4 and 18 days after SE as indicated. The hippocampal proteins were separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein amounts were determined by quantitative immunoblotting. The values are represented relative to those of control mice. Statistical analysis was performed with one-way ANOVA followed by Tukey-Kramer's honest significant difference *post-hoc* tests. * $p < 0.05$, ** $p < 0.01$ compared with the control.

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