

Frequent spontaneous seizures followed by spatial working memory/anxiety deficits in mice lacking sphingosine 1-phosphate receptor 2

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ABSTRACT

The diverse physiological effects of sphingosine 1-phosphate (S1P) are mostly mediated by its five cognate G protein-coupled receptors, S1P₁–S1P₅, which have attracted much attention as future drug targets. To gain insight into S1P₂-mediated signaling, we analyzed frequent spontaneous seizures in S1P₂-deficient (S1P₂^{−/−}) mice obtained after several backcrosses onto a C57BL/6N background. Full-time video recording of 120 S1P₂^{−/−} mice identified 420 seizures both day and night between postnatal days 25 and 45, which were accompanied by high-voltage synchronized cortical discharges and a series of typical episodes: wild run, tonic-clonic convulsion, freezing, and, occasionally, death. Nearly 40% of 224 S1P₂^{−/−} mice died after such seizures, while the remaining 60% of the mice survived to adulthood; however, approximately half of the deliveries from S1P₂^{−/−} pregnant mice resulted in neonatal death. In situ hybridization revealed exclusive *s1p2* expression in the hippocampal pyramidal/granular neurons of wild-type mice, and immunohistochemistry/microarray analyses identified enhanced gliosis in the whole hippocampus and its neighboring neocortex in seizure-prone adult S1P₂^{−/−} mice. Seizure-prone adult S1P₂^{−/−} mice displayed impaired spatial working memory in the eight-arm radial maze test and increased anxiety in the elevated plus maze test, whereas their passive avoidance learning memory performance in the step-through test and hippocampal long-term potentiation was indistinguishable from that of wild-type mice. Our findings suggest that blockade of S1P₂ signaling may cause seizures/hippocampal insults and impair some specific central nervous system functions.

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1. Introduction

Sphingosine 1-phosphate (S1P) is a potent (lyso)phospholipid mediator that is produced from activated platelets, mast cells, and many other cell types, and elicits diverse biological effects on most types of cells, including hippocampal neurons [1]. S1P is known to act as both an extracellular mediator and an intracellular second messenger, but most of its effects are mediated by its five cognate G protein-coupled receptors, S1P₁–S1P₅ (reviewed in [2–4]). The universal expression of S1P₁–S1P₃ receptors in most cell types/tissues and a lack of receptor subtype-specific agonists/antagonists have encouraged

many researchers to generate mice deficient in one or several of all five S1P receptors [5–12].

So far, four independent research groups have generated S1P₂-deficient (S1P₂^{−/−}) mice [6,8–10]. MacLennan et al. were the first to report S1P₂^{−/−} mice that display spontaneous, sporadic, and occasionally lethal seizures between postnatal weeks 3 and 7 [6]. Electroencephalographic abnormalities were identified both during and between the seizures, and hyperexcitable neocortical pyramidal neurons were detected without any apparent anatomical alteration [6]. Thereafter, Kono et al. and we reported that S1P₂^{−/−} mice are apparently normal and free of such seizures [8,10]. Finally, Du et al. recently mentioned that S1P₂^{−/−} mice occasionally have lethal seizures around weaning age [9]. Such phenotypic alteration could be due to mouse strain differences because the four groups analyzed S1P₂^{−/−} mice with different genetic backgrounds: 129/SvEvBrd × C57BL/6 (albino) [6], (129/Sv × 129/J)F₁ (originated from R1 ES cells) × C57BL/6N [8],

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129/SvEvTacrBR (originated from TC1 ES cells) × C57BL/6 [10], and C57BL/6 (fourth backcross generation: N4) [9], respectively.

After the initial analysis of $S1P_2^{-/-}$ mice on the mixed background [8], we continued to backcross onto C57BL/6N and then unexpectedly observed frequent spontaneous seizures in our $S1P_2^{-/-}$ colony in the seventh backcross generation (N7). There is accumulating evidence that $S1P_2$ signaling participates in various aspects of cardiovascular physiology/pathology [13]. Our recent studies using $S1P_2^{-/-}$ mice suggest that $S1P_2$ antagonists may have the potential to improve fibrosis after liver injury [14] and ameliorate diabetic conditions [15]. Another group recently reported that blockage of $S1P_2$ signaling attenuates atherosclerotic plaque formation in aortas and inhibits modified low-density lipoprotein accumulation in macrophages of mice [16]. In this study, we characterized seizures and analyzed central nervous system insults in $S1P_2^{-/-}$ mice to evaluate the physiological roles played by $S1P$ in the brain as well as the possible side effects of $S1P_2$ antagonism.

2. Materials and methods

2.1. Mice

$S1P_2^{-/-}$ mice were generated as previously described [8]. Heterozygous $S1P_2^{+/-}$ mice were backcrossed onto C57BL/6N (Clea Japan, Tokyo, Japan) for seven generations, and all mice used in this study were obtained from intercrosses between $S1P_2^{+/-}$ (N7) females and males. Mice were fed standard chow/water ad libitum (except during the eight-arm radial maze test) and kept under a 12-hour light/dark cycle (8 AM/8 PM) in an air-conditioned room. Animal protocols were approved by the animal care and use committees of Gunma, Yamagata, and Musashino universities and the Tokyo Institute of Psychiatry.

2.2. Full-time video recording

Mice were housed in independent cages and monitored by recording using a GR-DF590 digital video camera equipped with a 1.33-megapixel CCD sensor (JVC). Movies were processed with an iMovie program (Apple) and thoroughly checked by a single experimenter (N.A.).

2.3. Electroencephalography

Four-week-old wild-type (WT) and $S1P_2^{-/-}$ males were implanted with EEG electrodes, and electrical activity was recorded using the buffer amplifier [17,18]. Briefly, surgical procedures for chronic implantation of electrodes were carried out under anesthesia with pentobarbital (65 mg/kg, ip). Stainless-steel screw electrodes (1 mm in diameter) were placed on the temporal (Br − 2.5 mm, L 2.5 mm), occipital (Br − 3.5 mm, L 1.5 mm), parietal (Br − 1 mm, L 1.5 mm), and frontal (Br + 1.5 mm, L 1.5 mm) cortices. Another screw electrode was set on the cerebellum as the reference. Lead wires from the electrodes were connected to a small electrical socket. The electrodes and the socket were attached to the skull with dental cement. After a 3-day recovery period after surgery, EEG was recorded for about 8 hours in a sound-attenuated experimental box. The data were amplified 1000 times (time constant, 0.3 seconds; low-pass filter, 100 Hz; AB-621G; Nihon Kohden, Tokyo, Japan) and monitored on a computer to check the EEG changes (Spike 2; Cambridge Electronics Design, Cambridge, UK).

2.4. In situ hybridization

Mouse $s1p_2$ genomic DNA [19] was used as a PCR template to clone the amino-terminal-end 320-bp $s1p_2$ open reading frame sequences. The amplified $s1p_2$ cDNA was cloned into *Bam*HI/*Xho*I

sites of a pBluescript SK(+) vector (Stratagene); the plasmid was linearized with either *Xho*I or *Bam*HI, and sense or antisense digoxigenin-labeled riboprobes were synthesized using T3 or T7 RNA polymerase. Sagittal sections of 4-week-old WT males were cut (20 μ m), hybridized with either antisense or sense (as a control) probes, and visualized using an alkaline phosphatase-conjugated anti-digoxigenin antibody as described previously [20].

2.5. Immunohistochemistry

Sagittal brain sections of 9-week-old WT and $S1P_2^{-/-}$ males/females were cut at 10 μ m with a CM1900 cryostat (Leica), and stained with anti-neuronal nuclei (NeuN) mouse monoclonal (1:1000; Chemicon, Catalog No. MAB377) and anti-gliar fibrillary acidic protein (GFAP) rabbit polyclonal antibodies (no dilution; DakoCytomation, Catalog No. N1506). Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG antibodies (Invitrogen) were used as secondary antibodies at a dilution of 1:500. The slides were coverslipped with PermaFluor (Beckman Coulter) and observed with an LSM 510 META laser-scanning microscope fitted with a Plan-Neofluar 10 \times /0.3 NA objective (Carl Zeiss).

2.6. RNA isolation and microarray

Neocortices and hippocampi isolated from 9-week-old WT and $S1P_2^{-/-}$ (five males and five females each) were homogenized in Trizol (Invitrogen), and total RNA was extracted and purified using an RNeasy Mini Kit (Qiagen). Reverse transcription, replication of DNA, biotin labeling of RNA during in vitro transcription, hybridization, washing, and scanning of the Affymetrix GeneChip Murine Genome U74Av2 array that represents 12,488 probe sets were carried out as described previously [21]. Microarray Suite 5.0 software (Affymetrix) was used to scan 20 arrays (one array per mouse per brain region) and analyze the data; it determines a detection call, in which P values of <0.04, 0.04–0.06, and >0.06 are defined as cutoff points for present, marginal, and absent calls, respectively. The data obtained were deposited in Gene Expression Omnibus under Accession No. GSE7191. To ensure reliability, 7178 (57.5%) and 7179 (57.5%) of the 12,488 probe sets that gave hybridization signals with ≥ 2 absent or marginal calls among 10 arrays were excluded from further analyses of neocortical and hippocampal samples, respectively. Data for the remaining 5310 and 5309 probe sets, respectively, were considered reliable and named “validated.” The relative expression of validated probe sets was evaluated with the average expression level of 10 WT (either neocortical or hippocampal) samples set at 1. Differences were considered significant when $P < 0.05$ with an unpaired Student t test.

2.7. Behavioral tests

Locomotive activity was examined with the 5-minute open-field test as described previously [22]. Spatial working memory was evaluated with the eight-arm radial maze test [23]; the apparatus was made of gray polyvinyl chloride, and eight arms (25 \times 6 cm, with 20-cm-high transparent polyvinyl chloride walls) radiated from an octagonal platform (17.3 cm in diameter), elevated 60 cm off the ground. The maze was surrounded by a number of visuospatial cues, assumed to be perceivable by the animals while staying on the central platform, prior to each arm choice made, including doors, racks, posters, and ceiling textures, and was evenly illuminated by ceiling lamps. All arms were baited by placing a small food pellet (10 mg Kellogg’s Frosted Flakes) behind a low barrier, preventing the animal from seeing whether a specific arm was still baited or not. Three days prior to and throughout the trial, the mouse was deprived of food, but not water, from 3 PM to the following 11 AM, and thus body weight was kept at 80–90% of pretest body weight. Three days prior to the trial, the 9-week-old mouse also received

10 minutes of habituation per day at around 9 AM with fresh food at the end of each arm and free access to all arms. The actual trials were conducted for 16 consecutive days, one trial per day from 9 to 11 AM: the mouse was placed in the center and allowed a free choice of all eight arms. Entry into an arm was scored if the animal passed the entrance with all four limbs, and the number of errors was recorded each day. Each test was terminated after 10 minutes, after the animal had eaten all eight rewards, or after the animal had made 12 choices, whichever came first. An error was noted if the animal entered an arm previously visited or if it did not eat the reward; therefore, to perform well in this task, the animal had to store information continuously (in its spatial working memory) about which arms had already been visited during a particular trial and which arms had not.

Passive avoidance learning memory was tested with the step-through test [22], which uses the natural preference of mice for a dark environment. The apparatus consisted of bright and dark compartments. During the learning trial, mice were placed in the bright compartment and received an electric footshock (75 constant V and maximum 0.3 mA) on entering the dark compartment. During

the test period, starting on day 2, mice were placed in the bright compartment again for a maximum of 400 seconds at the same time each day for 2 or 4 days. The step-through latency, which indicates the time elapsed before the mouse enters the dark compartment, was recorded in the learning and testing trials as the performance of each mouse. Mice that did not enter the dark compartment were assigned a cutoff of 400 seconds.

Anxiety behaviors were examined using the elevated plus maze test [22], which consisted of four arms (25 × 6 cm), two enclosed by walls 20 cm high and two exposed, elevated 60 cm off the ground. Mice were placed in the center zone facing the same open arm, and their behavior was recorded. The amount of time spent in the open arms and the number of open-arm entries during the 5-minute test were determined as measures of anxiety.

2.8. Hippocampal slice electrophysiology

Hippocampal slices were prepared using standard procedures from 6-week-old WT and $S1P_2^{-/-}$ males, incubated for at least

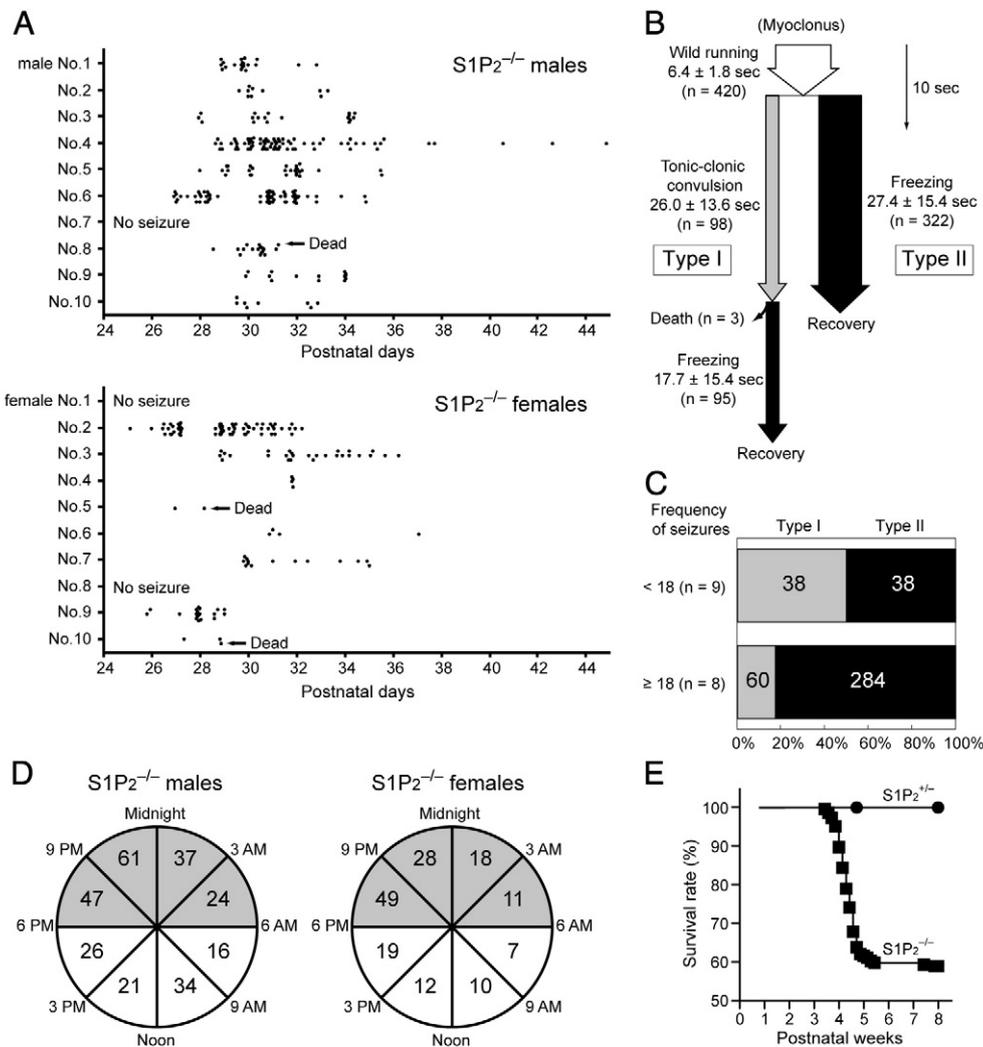


Fig. 1. Frequent spontaneous seizures in $S1P_2^{-/-}$ mice. (A) Frequency of wild runs. Full-time video recording of 20 $S1P_2^{-/-}$ mice (10 males and 10 females) identified 420 wild run episodes between P25 and P45. Each dot represents a single run episode. Three mice (1 male and 2 females) displayed no apparent run, and another 3 mice (1 male and 2 females) died from respiratory arrest during tonic convulsions. (B) Schematic representation of two types of seizures in $S1P_2^{-/-}$ mice. In type I, wild runs (that lasted for ~6.4 seconds) were followed by tonic-clonic convulsions (~26.0 seconds) and then freezing (~17.7 seconds). In the more frequent type II, wild runs were followed by freezing without apparent tonic-clonic convulsion phases. Three mice died after tonic convulsions in type I seizures. (C) Type I and II seizures in $S1P_2^{-/-}$ mice that had <18 (upper bar, $n = 9$) or ≥18 seizure episodes (lower bars, $n = 8$) by 8 weeks of age. (D) Timetable of the 420 wild runs in $S1P_2^{-/-}$ (left, males; right, females). Mice were kept under a 12-hour dark/light cycle (lights on 6 AM–6 PM). (E) Kaplan–Meier survival analysis of $S1P_2^{+/+}$ (circles, $n = 121$) and $S1P_2^{-/-}$ (squares, $n = 224$) mice. Mice were genotyped at 2 weeks of age and their survival status was monitored up to 8 postnatal weeks. Ninety of 224 (40.2%) $S1P_2^{-/-}$ mice were found dead between postnatal weeks 3.5 and 6 (seizure-prone period), whereas all $S1P_2^{+/+}$ mice survived. The difference was significant; $P < 0.0001$, in the log-rank test.

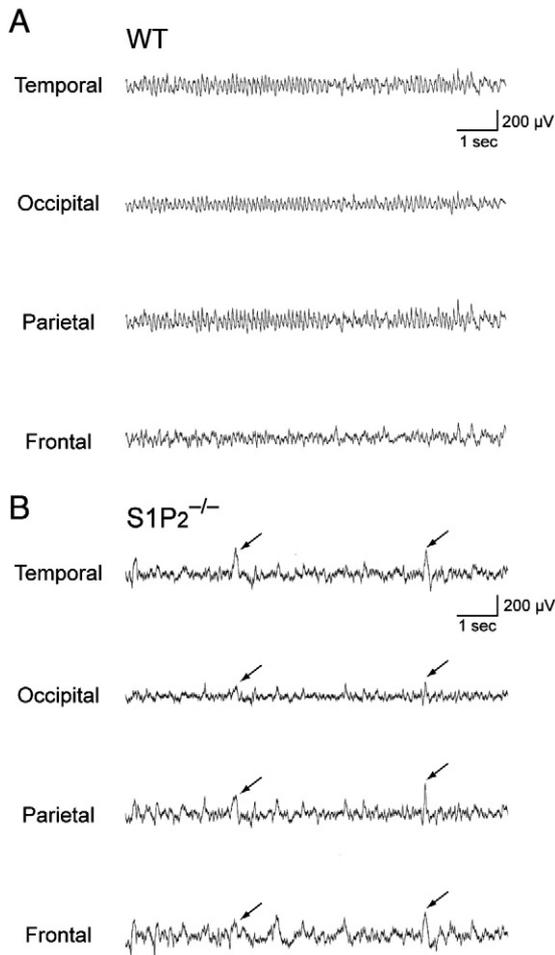


Fig. 2. Synchronized high-voltage discharges in cerebral cortical regions of $S1P_2^{-/-}$ mice. Electroencephalograms were recorded on the cerebral cortices of 4-week-old WT (A) and seizure-prone $S1P_2^{-/-}$ (B) males (during interictal periods). Synchronized (over temporal, occipital, parietal, and frontal lobes), high-voltage discharges were observed in $S1P_2^{-/-}$ mice (B, arrows), but not their littermate WT mice. Only the WT recording shows theta activity.

2 hours, and then transferred to a submersion-type recording chamber [24]. Slices were perfused with a standard artificial cerebrospinal fluid (119 NaCl, 2.5 KCl, 4.0 CaCl₂, 4.0 MgSO₄, 1.0 NaH₂PO₄, 26.2 NaHCO₃, 11 glucose, and 0.1 picrotoxin, in mM) that was bubbled continuously with 95% O₂/5% CO₂ at 28 °C. The Schaffer collateral/commissural pathway was stimulated with a stimulating electrode placed in the stratum radiatum, and field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 region. Long-term potentiation (LTP) was induced by two 100-Hz, 1-second tetani.

Table 1
Marked lethality in neonates born to $S1P_2^{-/-}$ dams.

Parental genotype		Number of surviving litters ^a	Number of extinct litters ^b	Lethality	Average size of surviving litters	Number of offspring in surviving litter			
Female	Male					Total	+/+	+/-	-/-
+/+	+/+	25	2	8.0%	8.2 ± 2.9	206	206	–	–
+/–	+/–	45	7	13.5%	5.4 ± 2.7	242	75	119	48
+/–	–/–	70	24	25.5%	5.8 ± 2.5	408	–	234	174
–/–	+/–	16	18	52.9%	5.0 ± 2.0	80	–	50	30
–/–	–/–	14	26	65.0%	5.8 ± 2.1	81	–	–	81

^a Surviving litters are litters in which all neonates that survived beyond P2.5 grew to 3 weeks of age. Litter sizes were counted and mice were genotyped at 3 weeks of age.

^b Extinct litters are litters in which all neonates were found dead by P2.5.

3. Results

3.1. Frequent spontaneous seizures in juvenile $S1P_2^{-/-}$ mice

We observed frequent spontaneous seizures characterized by wild running in $S1P_2^{-/-}$ mice on a C57BL/6N (N7) background (Supplementary Movie S1—see Appendix). Full-time video monitoring of 20 $S1P_2^{-/-}$ mice (10 males and 10 females) during postnatal weeks 3–8 confirmed 420 wild runs in 17 mice (9 males and 8 females) between postnatal day 25 (P25) and P45 (Fig. 1A). Seizures were not apparent in three (1 male and 2 females) of 20 $S1P_2^{-/-}$ mice (Fig. 1A). Two females (Nos. 4 and 6) displayed only 4 seizure episodes, which highly contrasts with another three mice (males 4 and 6, and female 2) that had ≥ 70 episodes (Fig. 1A).

Spontaneous seizures were often, but not always, initiated with myoclonus (a spontaneous and brief twitching of muscles that appears in mice as a quick wiggle of whiskers) and followed by wild runs that lasted 6.4 ± 1.8 seconds (Fig. 1B). Overall, 76.7% of the 420 runs were followed by freezing (categorized as type II), and the remaining 23.3% were followed by tonic-clonic convulsions and then freezing (categorized as type I) (Fig. 1B). Although the majority of $S1P_2^{-/-}$ mice recovered from freezing, 3 mice (male 8 and females 5 and 10) died from respiratory arrest after the seizure associated with tonic-clonic convulsions (Figs. 1A and B). More than 80% of seizures observed in seizure-prone $S1P_2^{-/-}$ mice ($n = 8$) that exhibited ≥ 18 seizure episodes were type II (i.e., free of tonic-clonic convulsion), whereas only half of those in less seizure-prone $S1P_2^{-/-}$ mice ($n = 9$) were type II (Fig. 1C). Seizures were observed both day and night, although they more often occurred at the beginning of night (from 6 PM to midnight) than during the day (Fig. 1D). Ninety of 224 (40.2%) $S1P_2^{-/-}$ mice (both sexes) were found dead between postnatal weeks 3.5 and 6 (Fig. 1E) when severe seizures occurred most frequently (Fig. 1A).

3.2. Synchronized high-voltage discharges in $S1P_2^{-/-}$ cortex

Electroencephalograms were recorded on the cerebral cortices of 4-week-old WT and seizure-prone $S1P_2^{-/-}$ males (Figs. 2A and B, respectively). As the wild runs in $S1P_2^{-/-}$ were vigorous and accompanied by artifacts, recording was possible only during the interictal periods. Synchronized (over temporal, occipital, parietal, and frontal lobes), high-voltage ($>200 \mu V$) discharges were observed in $S1P_2^{-/-}$ (Fig. 2B) but not WT (Fig. 2A) mice.

3.3. Marked postnatal lethality in neonates born to $S1P_2^{-/-}$ dams

Despite frequent seizures during juvenescence, nearly 60% of $S1P_2^{-/-}$ males/females survived to adulthood. They appeared normal except for deafness/head tilt [25–27] and were fertile; however, marked postnatal lethality was observed in neonates born to $S1P_2^{-/-}$ dams (Table 1). Some $S1P_2^{-/-}$ dams seemed to lack the ability

of maternal nurturing, leading to complete neglect of their neonates. Of 40 deliveries from $S1P_2^{-/-}$, no neonatal survivors were found after P2.5 in 26 deliveries (65.0%), although 5.8 pups per litter (at average) survived in the remaining 14 litters (Table 1). No neonatal survivors were found after P2.5 in 18 of 34 deliveries (18/34 = 52.9%) from $S1P_2^{-/-}$ female \times $S1P_2^{+/+}$ male matings, which contrasts with deliveries from WT/ $S1P_2^{+/+}$ females that displayed 8.0–25.5% lethality (Table 1).

3.4. Enhanced gliosis in hippocampus and neocortex of seizure-prone adult $S1P_2^{-/-}$ mice

Impaired maternal nurturing could be due to irreversible central nervous system insults caused by frequent severe seizures during juvenescence. In situ hybridization identified restricted $s1p_2$ expression in the hippocampus of 4-week-old WT mice; $s1p_2$ was expressed in the hippocampal pyramidal neurons and the granular neurons of the dentate gyrus (Figs. 3A and C, respectively), but apparently not in other brain regions (data not shown). There was no $s1p_2$ expression in all brain regions of $S1P_2^{-/-}$ mice (data not shown). To investigate molecular events underlying seizure-induced insults in the $S1P_2^{-/-}$ brain, we examined the global gene expression using microarray in the two brain regions, neocortices and hippocampi, of 9-week-old WT and seizure-prone $S1P_2^{-/-}$ mice. Comparative analysis revealed that multiple probe sets were up- or downregulated significantly ($P < 0.05$) in either the neocortex or hippocampus of $S1P_2^{-/-}$ mice (Supplementary Table S1—see Appendix). Observed prominent features were the decreased expression of neuronal activity markers that belong to the AP-1 transcriptional factor (*Fos*, *Junb*, and *Fosb*)

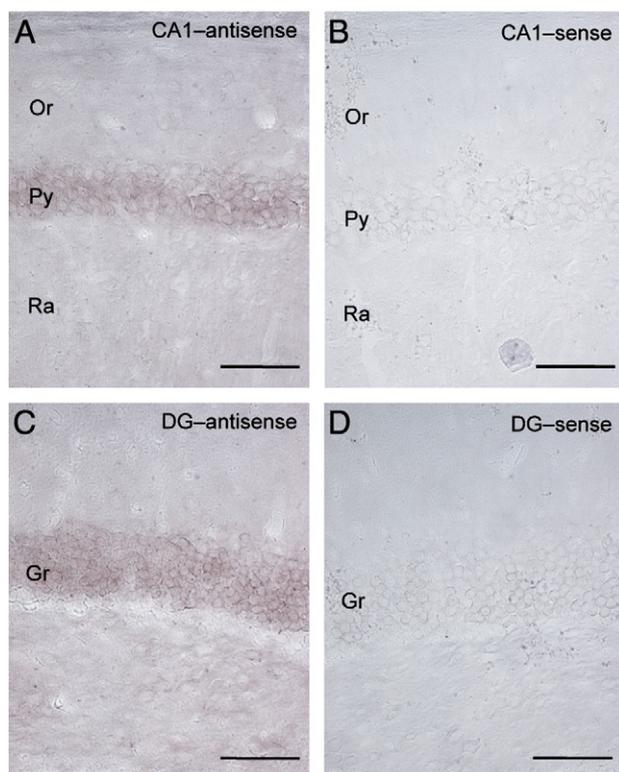


Fig. 3. Neuron-restricted $s1p_2$ expression in the hippocampus. In situ hybridization of hippocampal sections (A and B for CA1, C and D for dentate gyrus [DG]) from 4-week-old WT males with digoxigenin-labeled antisense (A, C) and sense (B, D, as background controls) $s1p_2$ riboprobes. Neuron-restricted $s1p_2$ expression was observed in the pyramidal cell layer (Py) (but neither stratum oriens [Or] nor stratum radiatum [Ra]) of CA1 as well as the granular cell layer (Gr) of DG. Bars: 100 μ m.

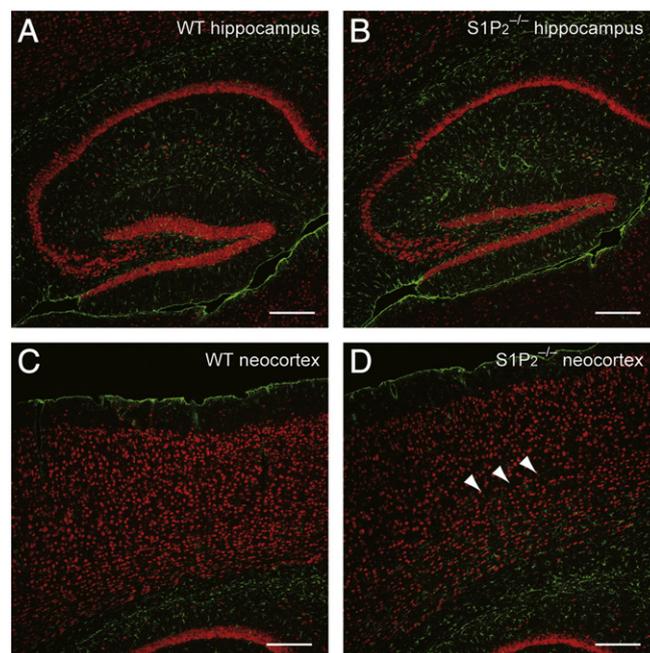


Fig. 4. Enhanced gliosis in the neocortex and the hippocampus of seizure-prone adult $S1P_2^{-/-}$ mice. Hippocampal (A, B) and neocortical (C, D) sections from 9-week-old WT (A, C) and seizure-prone (B, D) $S1P_2^{-/-}$ males were immunolabeled with anti-NeuN (red) and anti-GFAP (green) antibodies. Representative images from three mouse samples are shown. Enhanced gliosis was observed in the hippocampus (B) as well as the neocortical regions neighboring the stratum oriens of the CA1 (D, arrowheads). Bars: 100 μ m.

and the increased expression of *Gfap*, a gliosis marker, in both the neocortex and hippocampus of $S1P_2^{-/-}$ mice (*Fosb*, only in neocortex) (Supplementary Table S1—see Appendix). Double immunostaining of a neuronal cell marker with NeuN and GFAP revealed enhanced gliosis in the entire hippocampus of seizure-prone $S1P_2^{-/-}$ mice (Fig. 4B; compare with that of WT mice in Fig. 4A) as well as the part of the neocortex neighboring the stratum oriens of the CA1 area (Fig. 4D, arrowheads; compare with that of WT mice in Fig. 4C); such enhanced or expanded gliosis was not observed in seizure-free $S1P_2^{-/-}$ mice (data not shown).

3.5. Impaired spatial working memory and increased anxiety in seizure-prone adult $S1P_2^{-/-}$ mice

In the open-field test, there were no significant differences between 9-week-old WT and seizure-prone $S1P_2^{-/-}$ mice in both periods of movements and distances moved (data now shown); however, histological changes in the hippocampus may influence its major function, learning and memory. We examined spatial learning memory of seizure-prone adult $S1P_2^{-/-}$ mice using the positively reinforced eight-arm radial maze test and passive avoidance learning memory using the negatively reinforced step-through test. In the radial maze test, entry times into arms per minute did not differ between adult WT and $S1P_2^{-/-}$ mice throughout the trials (Fig. 5A), but the learning performance to find food pellets efficiently improved after several days of trials in adult WT mice but not in $S1P_2^{-/-}$ mice (Fig. 5B). In contrast, learning performance in the step-through test was indistinguishable, and LTP observed in the hippocampal slices was unaltered, between WT and $S1P_2^{-/-}$ mice (Figs. 5C and D, respectively) (WT, $176.8 \pm 12.6\%$ of baseline at 60 minutes after tetanus, $n = 8$; $S1P_2^{-/-}$, $162.4 \pm 3.9\%$, $n = 8$). In the elevated plus maze test, $S1P_2^{-/-}$ mice were more anxious (more prone to avoid entering and staying in the open area) than WT mice (Figs. 5E and F, respectively).

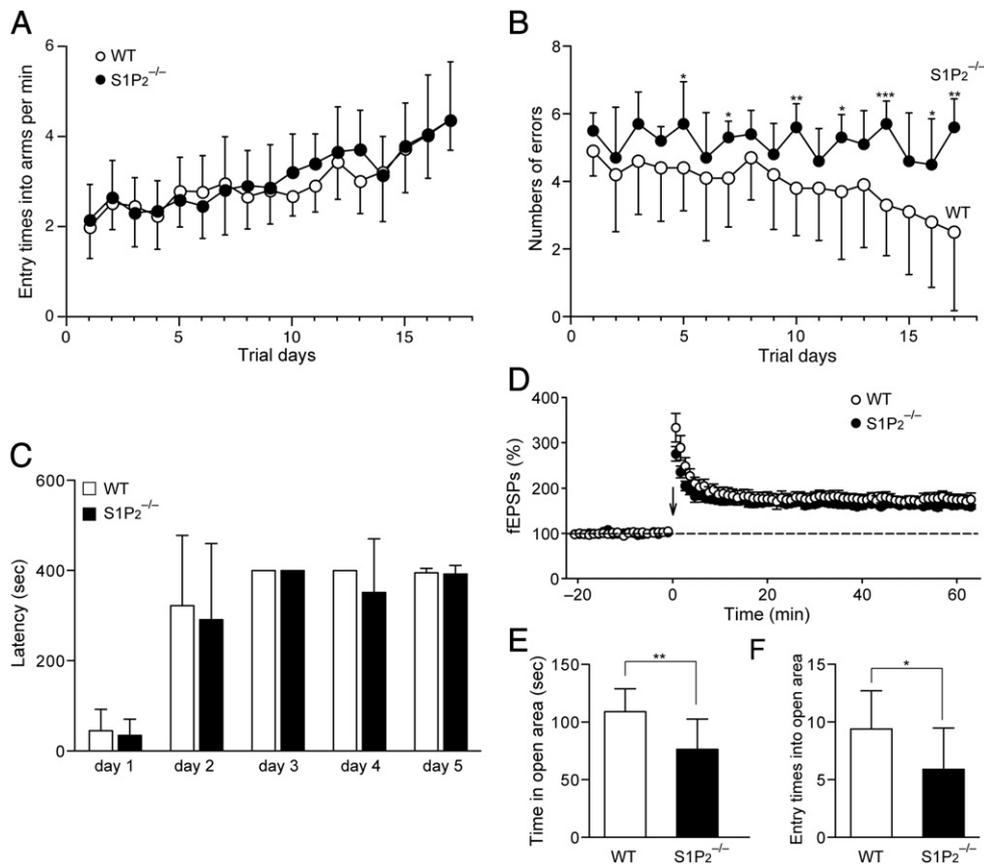


Fig. 5. Impaired and normal central nervous system functions in seizure-prone $S1P_2^{-/-}$ mice. (A, B) Normal locomotive activity but impaired spatial working memory in the eight-arm radial maze test. Numbers of entries into arms per minute (A) and errors in choosing baited arms (B) were scored until the mouse had eaten all 8 rewards or after the mouse made the first 12 choices, whichever came first. In the trial conducted for 16 consecutive days, daily performance was improved in 7-week-old WT males but not $S1P_2^{-/-}$ males ($n = 10$ each). (C) Normal passive avoidance learning memory in the step-through test. The mouse was placed in the bright compartment, and the time it took to enter the neighboring dark compartment (where it received an electric footshock) was measured with a cutoff of 400 seconds. In the trial executed for 5 consecutive days, 9- to 11-week-old WT ($n = 4$) and $S1P_2^{-/-}$ ($n = 6$) males performed similarly. (D) Normal hippocampal LTP in the $S1P_2^{-/-}$ slice. LTP was induced by two 100-Hz, 1-second tetani (arrow) in hippocampal slices from 6-week-old WT and $S1P_2^{-/-}$ males ($n = 8$ each). No significant difference was observed. (E, F) Altered anxiety behavior in the elevated plus maze test. Time (second) spent in open area was shorter (E) and entry times into open areas were fewer (F) in 7-week-old $S1P_2^{-/-}$ males than WT males ($n = 10$ each). $S1P_2^{-/-}$ males were slightly but significantly more anxious than WT males. Data are means \pm SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, unpaired Student t test.

4. Discussion

The present study demonstrated that the appearance of a seizure phenotype depends on the genetic background of the $S1P_2^{-/-}$ mice. Although seizures were not observed in $S1P_2^{-/-}$ mice on a 50:50 (129/Sv \times 129/J) F_1 :C57BL/6N background [8], apparent seizures were observed in 90% (18 of 20 in Fig. 1A) of $S1P_2^{-/-}$ mice with a much purer (N7, >99.2%) C57BL/6N background. We expect that some genes responsible for the onset of seizures have been replaced (from [129/Sv \times 129/J] F_1 origin to C57BL/6N origin) at some stage of backcrossing onto C57BL/6N. MacLennan et al. reported that the ~15% overall lethality rate in $S1P_2^{-/-}$ mice on a 50:50 129/SvEvBrd:C57BL/6 background dropped below 5% on a pure 129/SvEvBrd background [25]. The severity of seizures in our $S1P_2^{-/-}$ mice was the most pronounced in four independently generated populations of $S1P_2^{-/-}$ mice, our mice having the highest lethality rate (40% [Fig. 1E] compared with 15% [6,9] or 8–10% [9] on a C57BL/6 (N4) background) and lifetime frequency (~25 times on average [Fig. 1A] compared with <4 times [6]). This suggests that C57BL/6N mice carry several genes involved in the onset of seizures.

The existence of multiple S1P receptors with different functions underlies the variety of cellular responses elicited by S1P; all five $S1P_1$ – $S1P_5$ receptors were expressed in adult rat brain [28], and $s1p_1$ – $s1p_3$ and $s1p_5$ genes were expressed in adult mouse brain [8]. The S1P concentration in adult rat cerebrospinal fluid was reported to be 2.8 ng/mL (7.4 nM) [28], and the K_d value of $S1P_2$ receptor for S1P was estimated to be 20–27 nM [3]. Although $s1p_2$ transcript was

detectable in both the neocortex and hippocampus by reverse transcription PCR analysis [6], our *in situ* hybridization revealed $s1p_2$ expression only in pyramidal and granular neurons of the hippocampus (Figs. 3A and C). The $s1p_2$ gene was expressed in young, differentiating neuronal cell bodies and axons during early stages of rat central nervous system development [29], and hyperexcitable neocortical pyramidal neurons were detected by whole-cell patch-clamp recording in $S1P_2^{-/-}$ mice [6]. The $S1P_2$ receptor mediates various cellular signaling pathways, including the activation of adenylyl cyclase, phospholipase C, mitogen-activated protein kinase, phosphoinositide 3-kinase, and a small GTPase, Rho (but not Rac), some of which are common in $S1P_1$ – $S1P_5$ but others of which are unique to $S1P_2$ (i.e., Rac inhibition) [3]. $S1P/S1P_2$ -mediated signaling may contribute to the mediation of hippocampal excitability, and its complete absence during juvenescence may somehow trigger frequent spontaneous seizures originating from the hippocampus and then spreading over the neocortex (Fig. 2B), the final site of long-term storage for many kinds of information [30]. Indeed, enhanced gliosis was observed in the hippocampus (Fig. 4B, Supplementary Table S1—see Appendix) as well as its neighboring neocortex (Fig. 4D and Supplementary Table S1), and decreased expression of neuronal activity marker genes was observed in both the hippocampus and neocortex of $S1P_2^{-/-}$ mice (Supplementary Table S1); the former was previously observed in both the hippocampus and neocortex of the mice after kainate-induced seizures [21].

The association between hippocampal gliosis and spatial working memory/anxiety defects is intriguing. Previous studies mentioned

that hippocampal LTP was deficient when spatial learning memory performance was normal in endothelial nitric oxide synthase-deficient mice [31,32], indicating the dissociation between hippocampal LTP and spatial learning. Meanwhile, concomitant impairment of hippocampal LTP and performance in passive avoidance learning memory were observed in ubiquitin C-terminal hydrolase L1-deficient gracile axonal dystrophy (*gad*) mice [33], mice prenatally exposed to a cannabinoid agonist [34], and rats administered *N*-methyl-D-aspartate [35]. Therefore, our results—impaired spatial learning memory (Fig. 5B), normal passive avoidance learning memory (Fig. 5C), and normal hippocampal LTP (Fig. 5D)—agree with such previous studies.

The relationship between increased anxiety (in the elevated plus maze test) (Figs. 5E and F) and possible lack of maternal nurturing (Table 1) in $S1P_2^{-/-}$ mice remains unknown; the impaired oxytocin–dopamine signaling may be implicated [36]. The forced swimming test is widely used to test for depression-like symptoms in rodents; however, we could not use this test in our $S1P_2^{-/-}$ mice because they cannot swim due to vestibular defects [25,26].

In summary, the present study extensively characterized the seizures found in juvenile $S1P_2^{-/-}$ mice and revealed central nervous system insults remaining in the surviving adults: enhanced gliosis in the hippocampus/neocortex, impaired spatial learning memory, increased anxiety, and, possibly, impaired maternal nurturing. Our results caution that selective blockage of $S1P_2$ signaling may induce such central nervous system dysfunction in addition to auditory/vestibular defects [25–27].

Supplementary materials related to this article can be found online at doi:10.1016/j.yebeh.2011.09.002.

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