INTRODUCTION

The spatial K\(^+\) buffering by astrocytes removes excess extracellular K\(^+\) at synapses and transports them into regions of low K\(^+\) concentration such as blood vessels, regulating neuronal activities (Walz, 2000; Kofuji and Newman, 2004; Simard and Nedergaard, 2004; Butt and Kalsi, 2006). The K\(^+\) buffering currents are mediated by inwardly rectifying potassium (Kir) channels which are expressed in astrocytes (Tanemoto et al., 2000; Hibino et al., 2004; Kofuji and Newman, 2004; Simard and Nedergaard, 2004; Butt and Kalsi, 2006). These comprise Kir4.1 channels, homo-tetramers of Kir4.1 subunits, and Kir4.1/5.1 channels, hetero-tetramers of Kir4.1 and Kir5.1 subunits, which conduct large inward K\(^+\) currents at potentials negative to K\(^+\) equilibrium potential (Tanemoto et al., 2000; Ohno et al., 2007; Su et al., 2007; Furiutani et al., 2009). In addition, spatial K\(^+\) buffering is linked to glutamate uptake and/or aquaporin-4-mediated water transport by astrocytes (Nagelhus et al., 1999; Amiry-Moghaddam and Ottersen, 2006; Furutani et al., 2006; Simard and Nedergaard, 2004; Butt and Kalsi, 2006). These mutations caused drastic decreases in K\(^+\) currents mediated by Kir4.1 and Kir4.1/5.1 channels, suggesting that the impaired functioning of astrocytic Kir4.1 channels causes epileptic seizures by disrupting spatial K\(^+\) buffering. In addition, several SNPs of KCNJ10 have been shown to be associated with temporal lobe epilepsy (TLE) with febrile seizures (Heuser et al., 2010). Expressional analysis also revealed pathophysiological alterations in Kir4.1 expression in patients with TLE (Das et al., 2012; Heuser et al., 2012; Steinhäuser et al., 2012).
suggesting a potential involvement of Kir4.1 channels in TLE epileptogenesis. However, information on the modulatory role of Kir4.1 in the generation and/or development of TLE is still very limited.

In the present study, we performed expression analysis of Kir4.1 in a pilocarpine-induced rat model of TLE to explore the pathophysiological role of Kir4.1 channels in TLE epileptogenesis. The expressions of Kir5.1 and Kir2.1, other Kir subunits expressed in astrocytes, were also evaluated for comparison.

**RESULTS**

**PILOCARPINE-INDUCED TLE MODEL**

All the TLE rats (N = 11) used herein experienced pilocarpine (350 mg/kg, i.p.)-induced status epilepticus (repeated and sustained tonic seizures) and showed spontaneous seizures (i.e., wild running/jumping and GTCS; seizures) 7–8 weeks after the pilocarpine treatment. The animals, which were given pilocarpine but did not experience status epilepticus and any seizure activity thereafter (7–8 weeks), were used as the control (N = 11). Four and seven animals in each group were subjected to Western blot analysis and immunohistochemical analysis, respectively.

**WESTERN BLOT ANALYSIS**

As reported previously (Connors et al., 2004; Seifert et al., 2009; Harada et al., 2013), Kir4.1 was detected primarily as a tetramer (∼160 kDa) in all brain regions examined in TLE and control rats (Figure 1A). Two-way ANOVA revealed no significant interaction [F(1, 60) = 1.61, P = 0.13], but significant main effects of groups [F(1, 60) = 25.24, P < 0.01] and regions [F(9, 60) = 10.80, P < 0.01]. Expression levels of Kir4.1 were relatively high in the striatum (St) and pons/medulla oblongata (P/MO). As compared to control animals, TLE rats showed significantly higher Kir4.1 levels in the frontal cortex (fCx, P < 0.05), occipito-temporal cortex (otCx; P < 0.01), hippocampus (Hpc), thalamus (Th), midbrain (Mid), and cerebellum (Cer) [i.e., parieto-temporal cortex (ptCx), hippocampus (Hpc), thalamus (Th), midbrain (Mid), and cerebellum (Cer)] remained unaltered.

In contrast to Kir4.1, Kir5.1 and Kir2.1 subunits were detected mainly as monomers (Kir5.1: 50 kDa, Kir2.1: 45 kDa) in all 10 regions (Figure 1A). Levels of Kir5.1 were relatively high in the ptCx and Mid while the Kir2.1 levels were high in the Mid and low in the cerebral cortices and Cer (Figures 1C,D). Analysis of Kir5.1 expression showed only a significant main effect of regions [F(9, 60) = 7.97, P < 0.01] without a significant interaction [F(9, 60) = 0.32, P = 0.96] or a main effect of groups [F(1, 60) = 0.77, P = 0.38]. Thus, no significant differences in the expression levels of Kir5.1 were observed between TLE and control rats in all 10 regions (Figure 1C). On the other hand, analysis of Kir2.1 expression revealed significant main effects of groups [F(1, 60) = 7.93, P < 0.01] and regions [F(9, 60) = 13.9, P < 0.01] without a significant interaction [F(9, 60) = 1.24, P = 0.29]. Among 10 regions, only the Kir2.1 level in the St was significantly (P < 0.01) higher in TLE than in control rats (Figure 1D).

**IMMUNOHISTOCHEMICAL ANALYSIS FOR KIRA.1 EXPRESSION**

Since Western blot analysis revealed that pilocarpine-induced TLE rats showed elevated Kir4.1 expression in the fCx and otCx, we further conducted immunohistochemical analysis for Kir4.1 expression using frontal (Bregma + 1.68 mm level) and occipito-temporal (Bregma −3.00 mm level) brain slices (Figure 2A).

With regard to the expression patterns of Kir4.1 immunoreactivity (IR), we have previously shown that Kir4.1 was primarily stained in astrocytes which typically show a stellate-shape and were...
specifically co-stained with glial fibrillary acidic protein (GFAP; an astrocyte marker; Harada et al., 2013; also see Figure A1 in Appendix). Although Kir4.1-IR was also found in a small population of round-shaped (small) cells, which might possibly represent oligodendrocyte precursor cells (Maldonado et al., 2013), we omitted them from the analysis and solely counted the stellate-shaped astrocytes probe with anti-Kir4.1 antibody. In addition, to evaluate changes in the total number of astrocytes per se and the Kir4.1 expression ratio relative to the total number of astrocytes, we also performed immunohistochemical analysis of GFAP using paired successive slices obtained from the same animal.

In accordance with previous studies (Connors et al., 2004; Seifert et al., 2009; Harada et al., 2013), Kir4.1 was mostly expressed in stellate-shaped cells (Figure 2B). Two-way ANOVA revealed significant interaction groups × regions \( F(21, 264) = 1.91, P < 0.05 \) and significant main effects of groups \( F(1, 264) = 410.45, P < 0.01 \) and regions \( F(21, 264) = 3.50, P < 0.01 \). In pilocarpine-induced TLE rats, Kir4.1 expression was significantly elevated in all brain regions examined [dentate gyrus of the Hpc (DG) and dorsomedial St (dmST): \( P < 0.05 \), other regions: \( P < 0.01 \)] except for the agranular insular cortex dorsal part (AID; Figures 2–4). The number of Kir4.1-IR-positive astrocytes increased two to four times the control levels in TLE animals and these changes were prominent in the sensory cortex (SC), lateral St, and amygdala (Figures 3A and 4A). In addition, the number of GFAP-IR-positive astrocytes per se also increased in pilocarpine-induced TLE rats (Figures 3B and 4B). Analysis
of GFAP expression showed significant main effects of groups \([F(1, 264) = 335.16, P < 0.01]\) and regions \([F(21, 264) = 6.26, P < 0.01]\) without a significant interaction \([F(21, 264) = 0.86, P = 0.65]\). The numbers of GFAP-IR-positive cells in all 22 brain regions examined were significantly \((P < 0.01)\) parts of the medial amygdaloid nucleus, the posteromedial SC \((P < 0.01)\), ventrolateral ST \((P < 0.01)\), and Pir \((P < 0.05; \text{Figures 3C and 4C})\). Two-way ANOVA revealed significant interaction \([F(1, 264) = 1.78, P < 0.05]\) and significant main effects of groups \([F(1, 264) = 12.82, P < 0.01]\) and regions \([F(21, 264) = 2.36, P < 0.01]\). The relative Kir4.1 expression ratios in astrocytes were 0.3–0.8 in most regions of the brain in the control rats. We then compared the Kir4.1 expression ratios relative to the number of astrocytes (Kir4.1-IR-positive cells/GFAP-IR-positive cells). Two-way ANOVA revealed significant interaction \([P < 0.01]\) parts of the medial amygdaloid nucleus, the posteromedial cortical amygdaloid nucleus (PMCo, \(P < 0.01)\), and regions \([P < 0.01]\). These changes were region-specific and significant increases were observed in the posteroventral (MePV, \(P < 0.05)\) and posterodorsal (MePD, \(P < 0.01)\) parts of the medial amygdaloid nucleus, the posteroventral (MePV, \(P < 0.01)\), and regions \([P < 0.01]\) without a significant interaction [\(F(21, 264) = 1.78, P = 0.05\), other regions: \(P = 0.01\)].

**DISCUSSION**

Temporal lobe epilepsy is the most common type of partial complex seizure in adulthood (Hausser et al., 1996; Wieser, 2004). The main features of TLE include: (1) localization of seizure foci in the limbic structures (e.g., Hpc and amygdala), (2) existence of a "latent period," a seizure-free time interval following the initial precipitating injury, (3) incidence of mesial sclerosis leading to atrophy (e.g., neuronal loss and gliosis) in the limbic structures (Mathern et al., 1997; Bartolomei et al., 2005; Curia et al., 2008). The pilocarpine-induced TLE model shares important features of human TLE such as: (1) presence of a latent period followed by spontaneous recurrent seizures, (2) occurrence of wide spread brain injuries resembling human TLE, (3) similarity of drug responses to human TLE (e.g., relatively resistant to conventional antiepileptics; Leite et al., 1990; Cavalcione et al., 1991; Glen et al., 2002; Loscher, 2002; Wieser, 2004; Chakir et al., 2006; Curia et al., 2008). The present study demonstrated for the first time that expression of astrocytic Kir4.1 channels mediating spatial \(K^+\) buffering was markedly elevated in a pilocarpine-induced TLE model. The elevation of Kir4.1 expression in the TLE model was characterized by the following points, (1) subunit-specificity for Kir4.1, (2) a partial association with an increase in the number of astrocytes (i.e., astrogliosis) and (3) the most prominent elevation in the amygdala.

In this study, Western blot analysis revealed that the pilocarpine-induced TLE model exhibits a subunit-specific increase in the Kir4.1 expression with negligibly affecting the level of Kir5.1 and Kir2.1 subunits. Kir5.1 subunits, like Kir4.1, are expressed in astrocytes and form heteromeric Kir4.1/5.1 channels with Kir4.1, mediating \(K^+\) buffering (Tanemoto et al., 2000; Hibiino et al., 2004; Kofuji and Newman, 2004). In contrast, Kir2.1 subunits are predominantly expressed in neurons to regulate the resting membrane potential while several reports show that astrocytes also express Kir2.1 to some degree in several brain regions (e.g., Pir and olfactory bulb, Howe et al., 2008; Kang et al., 2008). Our results suggest that, among astrocytic Kir channels, Kir4.1 channels play the most important role in modulating TLE epileptogenesis.

Elevation of Kir4.1 expression in the pilocarpine-induced TLE model was widely spread throughout brain regions examined and these changes were generally associated with an increase in the number of astrocytes, which was probably due to astrogliosis following status epilepticus-induced brain injury (Leite et al., 1990; Cavalcione et al., 1991; Borges et al., 2003; Curia et al., 2008). Although astrogliosis may also contribute to epileptogenesis, it can compensate abnormal discharges and promote tissue repair. Astrocytes can reduce abnormal neural excitation.
FIGURE 4 | Topographical expression of Kir4.1 and GFAP in the basal ganglia and limbic regions of pilocarpine-induced TLE rats. (A,B) Number of Kir4.1- or GFAP-positive cells. (C) Relative Kir4.1 expression ratios in astrocytes. A pair of successive slices in each region from the same animal was stained with anti-Kir4.1 or anti-GFAP antibody. The Kir4.1 expression ratios were calculated as the ratios of Kir4.1-positive astrocytes relative to the total number of astrocytes (Kir4.1-positive cells/GFAP-positive cells) in each animal: dmST, vmST, dlST, and vlST, dorsomedial, ventromedial, dorsolateral, and ventrolateral striatum, respectively; AcbC and AcbSh, core and shell regions of the nucleus accumbens, respectively; MePV and MePD, medial amygdaloid nucleus, posteroverentral and posterodorsal part; BLP, basolateral amygdaloid nucleus, posterior part; BMP, basomedial amygdaloid nucleus, posterior part; PMCo, posteromedial cortical amygdaloid nucleus; CA1, CA3, and DG, CA1, CA3, and dentate gyrus of the hippocampus. Each column represents the mean ± SEM of seven animals. * P < 0.05, ** P < 0.01, significantly different from control rats.

by spatial buffering of potassium and by taking up synaptically released glutamate. In addition, they can secrete growth factors (e.g., glial cell line-derived neurotrophic factor [GDNF]) and nerve growth factor (NGF) and cytokines (e.g., TNF-α) that mediate neuronal survival, axonal/dendritic sprouting, and homeostatic plasticity (Borges et al., 2003; Fellin, 2009). Thus, the up-regulation of Kir4.1 associated with status epilepticus-induced astrogliosis might negatively regulate the TLE epileptogenesis by normalizing extracellular K⁺ ([K⁺]ₒ) and glutamate ([glutamate]ₒ). Furthermore, significantly higher Kir4.1
expression ratios relative to the number of astrocytes (Kir4.1-IR-positive cells/GFAP-IR-positive cells) were observed region-specifically in the amygdaloid nuclei (i.e., MePV, MePD, and PMCo). These results illustrate the important role of amygdalear Kir4.1 channels in modifying status epilepticus-induced epileptogenicity in TLE. Since deficit or knockdown of astrocytic Kir4.1 channels is known to impair K+ and glutamate-uptake into astrocytes and facilitate seizure generation (Ujiki et al., 2007; Kucheryavykh et al., 2007; Bockenhauer et al., 2009; Scholl et al., 2009; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010), up-regulation of Kir4.1 channels in the pilocarpine TLE model seemed to occur as a compensatory mechanism to the limbic hyperexcitability in TLE epileptogenesis. Indeed, the medial amygdaloid and cortical amygdaloid nuclei are known to be closely linked to kindling epileptogenesis and human epileptic disorders including TLE (Hosford et al., 1995; Morimoto et al., 2004). Although it is known that pilocarpine-induced status epilepticus causes neural damage, sclerosis, and rewiring not only in the amygdala, but also in the Hpc, changes in the relative Kir4.1 expression ratios were not significant in the Hpc (i.e., CA2). This may be due to the relatively low expression level of Kir4.1 in the Hpc as compared to other brain regions (see Figure 2).

Evidence is accumulating that dysfunction of astrocytic Kir4.1 channels is causative of seizure activity generation. Specifically, loss-of-function mutations in human Kir4.1 gene (KCNJ10) cause the EAST syndrome, including GTC seizures and ataxia (Bockenhauer et al., 2009; Scholl et al., 2009; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010). It is also suggested that the down-regulation of Kir4.1 expression in the amygdala is related to seizure induction in an animal model of GTC seizures (Harada et al., 2013). Furthermore, recent studies showed the down-regulation and/or impaired functioning of Kir4.1 channels in specimens from patients with TLE (Das et al., 2012; Heuser et al., 2013) and in specimens from patients with TLE (Das et al., 2012; Heuser et al., 2013; Kucheryavykh et al., 2007; Bockenhauer et al., 2009; Scholl et al., 2009; Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010), suggesting a close relationship of Kir4.1 to human TLE. The present results (Kir4.1 up-regulation) in the pilocarpine-induced TLE model, however, were different from the findings of Kir4.1 expression (Kir4.1 down-regulation) in patients with TLE. Although the reasons for this discrepancy are currently uncertain, it may result from the difference in the etiological basis between human TLE and pharmacologically evoked seizure. In fact, we also observed that Kir4.1 expression in the paralimbic cortex was gradually increased during the kindling development induced by pentyleneetrazole (Mukai et al., 2013). Alternatively, it may be due to the temporal changes in Kir4.1 expression. Since the present study analyzed the Kir4.1 expression shortly after the occurrence of spontaneous seizures, the down-regulation of Kir4.1 may occur at a more advanced (delayed) stage in the pilocarpine-induced TLE model. Indeed, a recent study showed that Kir4.1 expression was down-regulated by local inflammatory events after TLE-associated brain injury, implying that the down-regulation of Kir4.1 could be a consequence, and not a primary cause, of seizures (Zarolo et al., 2012). Further studies are required to delineate the time course of the Kir4.1 expression changes and the mechanisms underlying the Kir4.1 up-regulation in the pilocarpine-induced TLE model.

In conclusion, we performed expression analysis of Kir4.1 in a pilocarpine-induced rat model of TLE to explore the pathophysiological role of Kir4.1 channels in epileptogenesis. Western blot analysis revealed that Kir4.1 levels of TLE rats under an interictal state were significantly increased in the cerebral cortex, St, and Ht while the levels of other Kir subunits, Kir2.1, were unaltered. Immunohistochemical analysis demonstrated that TLE rats showed a widespread elevation in Kir4.1 expression which accompanied an increase in the number of astrocytes per se. In addition, the Kir4.1 expression ratio relative to the increase in the astrocyte number was also elevated region-specifically in the amygdaloid nuclei in a pilocarpine TLE model. The present findings suggest that astrocytic Kir4.1 channels play a modulatory role in TLE epileptogenesis, possibly by acting as an inhibitory compensatory mechanism. Further studies using patch-clamp and/or microdialysis techniques are necessary to delineate the functional alterations (e.g., changes in Kir4.1-mediated potassium currents, extracellular levels of K+ and glutamate) of up-regulated Kir4.1 channels in the TLE model.
WESTERN BLOT ANALYSIS

Temporal lobe epilepsy rats under interictal conditions (N = 4) or control rats (N = 4) were deeply anesthetized with pentobarbital (88 mg/kg, i.p.). The brain was then removed from the skull, chilled in ice-cold saline and dissected into the following 10 regions (ICx, pOCx, oCx, St, Hpc, Th, Ht, Mid, P/MO, and Cer). Brain samples were then homogenized in an ice-cold lysis buffer (pH 7.5) containing: (in mM) Tris 20, NaCl 150, MgCl2, 10, EDTA 1.0, EGTA 1.0, 1% Triton X-180, and a mixture of protease inhibitors (leupeptin, aprotinin, E-64, pepstatin A, bestatin, and 4-(2-aminoethyl) benzamidyl fluoride hydrochloride; Nacalai Tesque, Kyoto, Japan). The homogenate was centrifuged at 15,000 g for 30 min and the supernatant was stored at −80°C for the Western blot analysis.

Western blots were performed as published previously (Olino et al., 2009, Harada et al., 2013). Briefly, samples were incubated with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for 5 min at 95°C. Each sample (40 μg/lane) was then subjected to SDS-PAGE and separated proteins were transferred for 60 min to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was first incubated with a blocking solution containing 0.3–2% skim milk, 25 mM Tris, 150 mM NaCl, and 0.1% Tween 20 (pH 7.5) for 60 min, then with the corresponding primary antibody overnight (4°C), followed by a 60 min-incubation with the secondary antibody, a goat anti-rabbit IgG-HRP conjugate (1:2000, Santa Cruz Biotechnology) and a mouse anti-GFAP antibody (1:100, Progen) and mouse monoclonal antibodies against Kir5.1 (N-12; 1:400, Santa Cruz Biotechnology) and a goat anti-mouse IgG secondary antibody (1:400, Sigma-Aldrich) for 60 min with an avidin-biotinylated horseradish peroxidase complex ( Vectastain ABC Kit) for an additional 60 min. Kir4.1- and GFAP-IR was visualized by the diaminobenzidine-nickel staining method.

The number of Kir4.1- or GFAP-IR-positive cells was counted in a 350 × 350 μm2 grid laid over various regions of the brain (Figure 4), which included the following regions: the motor cortex (MC), SC, AID, ectothalamic-perirhinal cortex (Ect-PRh), Pir, dorsolateral St (dSt) and dmSt and ventromedial St (vmSt), core (AcbC) and shell (AcbSh) regions of the nucleus accumbens, MePV, MePD, basolateral amygdaloid nucleus posterior part (BLA), basomedial amygdaloid nucleus posterior part (BMP), PMCo, and CA1, CA3, and the DG of the Hpc. Relative expression rate of Kir4.1 was defined as a percentage of the number of Kir4.1-positive cells relative to that of GFAP-positive cells.

STATISTICAL ANALYSIS

All data are expressed as the mean ± SEM. Expression changes in Kir channel subunits determined by Western blot or immunohistochemical analysis were compared by two-way ANOVA followed by Tukey multiple comparison test. Differences were considered to be statistically significant for values of P < 0.05.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
APPENDIX

FIGURE A1 | Expressional patterns of Kir4.1 in the rat hippocampus. Typical photograph illustrating a double staining of Kir4.1 with GFAP in the hippocampal CA1 field. The hippocampal section was incubated anti-GFAP antibody (Progen, Heidelberg, Germany) for 24 h at 4°C and then incubated with a FITC (fluorescein isothiocyanate; green fluorescent) conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) or TRITC (tetramethylrhodamine-5- (and 6)-isothiocyanate; red fluorescence) conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich) to probe Kir4.1 and GFAP, respectively. Immunofluorescence images were obtained with a confocal laser scanning microscope. Scale bar: 50 μm. All the Kir4.1-immunoreactivity (IR)-positive cells with the stellate-shape were double-stained with anti-GFAP antibody (yellow in a merged picture). Kir4.1-IR was occasionally found in a few round-shaped cells with no GFAP-IR (also negative to a neuronal marker, neuronal nuclear antigen (NeuN)), which were omitted from the analysis.