GSK3β activity alleviates epileptogenesis and limits GluA1 phosphorylation


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A B S T R A C T

Background: Glycogen synthase kinase-3β (GSK3β) is a key regulator of cellular homeostasis. In neurons, GSK3β contributes to the control of neuronal transmission and plasticity, but its role in epilepsy remains to be defined.

Methods: Biochemical and electrophysiological methods were used to assess the role of GSK3β in regulating neuronal transmission and epileptogenesis. GSK3β activity was increased genetically in GSK3β-deficient mice and chemically depleted in brain slices and video electroencephalography on neuronal transmission and epileptogenesis induced by kainic acid were assessed by

Findings: Higher GSK3β activity decreased the progression of kainic acid induced epileptogenesis. At the biochemical level, higher GSK3β activity increased the expression of hyperpolarization-activated cyclic nucleotide-gated (HCN) channel 4 under basal conditions and in the epileptic mouse brain and decreased phosphorylation of the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluA1 at Serine 831 under basal conditions. Moreover, we found a significant correlation between higher inhibitory GSK3β phosphorylation at Serine 9 and higher activating GluA1 phosphorylation at Serine 845 in brain samples from epileptic patients.

Interpretation: Our data imply GSK3β activity in the protection of neuronal networks from hyper-activation in response to epileptogenic stimuli and indicate that the anti-epileptogenic function of GSK3β involves modulation of HCN4 level and the synaptic AMPA receptors pool.

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1. Introduction

Epilepsy results from abnormal excessive and synchronous activity of brain neuronal networks. Temporal lobe epilepsy (TLE) is one of the most common epilepsies in adults, involving pathological alterations in the hippocampal formation [1]. Structural abnormalities of the cerebral cortex (e.g., focal cortical dysplasia [FCD]) are increasingly recognized as important causes of severe, often intractable seizures.
Research in context

Evidence before this study

Glycogen synthase kinase-3 α (GSK3α) and GSK3β are key homeostatic kinases that regulate many cellular processes including neuronal synaptic plasticity. Aberrant neuronal plasticity often occurs in neuropathologies, e.g., epilepsy. However, the relationship between GSK3 and epileptogenesis is not clear and available data are contradictory. Based on our previous finding that mice deficient in neuronal GSK3β are characterized by poor survival in response to the proconvulsive drug, we hypothesized that GSK3β controls neuronal excitability and epileptogenesis.

Added value of this study

In this study, we provided evidence supporting our hypothesis. We showed that mice that overexpressed a constitutively active form of GSK3β in the brain developed fewer spontaneous seizures following status epilepticus. As for a potential mechanism we indicated changes in expression of HCN4 channel and activating phosphorylation of AMPA receptor subunit GluA1 which should result in decreased excitability of neuronal networks. Finally, in brain samples from epileptic patients, we found the correlation that higher activating GluA1 phosphorylation was accompanied by higher inhibitory GSK3β phosphorylation.

Implications of all the available evidence

Our study brings an important, clinically relevant observation that under certain circumstances GSK3β acts to slow down epileptogenesis. This data are also of vital importance due to the recent proposal of GSK3 inhibitors use for epilepsy treatment.

Epileptogenesis is the process that precedes the occurrence of epilepsy but continues after the diagnosis and includes epilepsy progression [2,3]. During this process several cellular and structural changes occur in response to the primary cause, including head trauma, status epilepticus, infections, and genetics. In humans, this latent period can take months or even years.

Exogenously triggered epileptogenesis can be mimicked in animals by electrical or chemical brain stimulation using chemocovulsants (e.g., kainic acid [KA]), which leads to an initial insult that triggers epileptogenesis [4]. These models are useful for identifying signaling pathways that potentially contribute to the development or manifestation of epilepsy. For example, phosphorylation of the glutamate GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) at Serine 831 (S831) and S845, increased the sensitivity to KA-induced seizures in mice [5]. Less is known, however, about the mechanisms of the resistance to epileptogenesis.

Glycogen synthase kinase-3α (GSK3α) and GSK3β are major homeostatic kinases that regulate a plethora of cellular processes [6,7] including neuronal transmission and synaptic as well as structural plasticity [8–12]. At the molecular level, GSK3β contributes to synaptic transmission, both pre- and postsynaptically, regulating the expression and trafficking of ion channels [13–15]. Little is known about the involvement of GSK3 kinases in epileptogenesis, while reported data are contradictory. For example, GSK3β phosphorylation at Serine 9 (S9), which inversely correlates with GSK3β activity, was reported to be increased [16] or decreased [17] in brain tissue that was resected from patients with TLE [16,17] and FCDIIa and FCDIIb patients [17]. Studies in various animal models also did not elucidate a clear relationship between GSK3 activity, status epilepticus and epileptogenesis [18–21] but GSK3β is considered to contribute to the development of epilepsy.

Considering the multilevel impact of GSK3 on different forms of synaptic plasticity and the profound effects of pentylenetetrazol on the survival of mice deficient in neuronal GSK3β, (GSK3β[−/−]) [11,22], we hypothesized that GSK3β controls neuronal responses to proconvulsive drugs and potentially impacts epileptogenesis. Thus, the present study was designed to test this hypothesis in the context of neuronal excitability and epileptogenesis. We showed that there was no significant difference in KA induced epileptiform discharges in acute hippocampal slices.

Table 1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Catalog no. (SRID)</th>
<th>Host</th>
<th>Dilution (Western blot)</th>
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**Fig. 1.** GSK3β regulates GluA1 phosphorylation and HCN4 expression in the mice brain. Hippocampi from wildtype \((n = 6)\) and GSK3β[S9A] \((n = 5)\) mice were isolated and lysed. (a) Western blot was used to determine the levels of GluA1, P-GluA1 (S845), P-GluA1 (S831), GluA2, GluN1, EEAT2, ErbB4, TrkB, HCN4, and GAD67. Tubulin is shown as a loading control. (b) Quantitative Western blot analysis results. Error bars indicate SEM. *\(p < .05\), **\(p < .01\), ***\(p < .001\) (two-way ANOVA significant interaction effect, \(p = 0.0001\), ***; two-way ANOVA with Bonferroni correction).

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### Table 2
Clinical characteristics of TLE-HS and FCD patients.

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<th>Case</th>
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<th>Age</th>
<th>Gender</th>
<th>Age of onset (years)</th>
<th>Seizure type</th>
<th>Number of seizures/month</th>
<th>AEDs</th>
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<td>Female</td>
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<td>FAS/FTBCS</td>
<td>32</td>
<td>LTG, TPM</td>
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<td>FIAS</td>
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<td>CBZ, CLB</td>
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</table>

**TLE-HS patients**

**FCD patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>Pathology ((adjacent to FCD2A))</th>
<th>Age</th>
<th>Gender</th>
<th>Age of onset (years)</th>
<th>Seizure type</th>
<th>Number of seizures/month</th>
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<td>Male</td>
<td>4</td>
<td>FIAS</td>
<td>36</td>
<td>VPA, LEV</td>
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</tbody>
</table>

\(\text{FIAS, focal impaired awareness seizure; FAS, focal aware seizures; FTBCS, focal-to-bilateral tonic-clonic seizure; LEV, levetiracetam; TPM, topiramate; LTG, lamotrigine; CNP, clonazepam; PHT, phenytoin; OXC, oxcarbazepine; CBZ, carbamazepine; CLB, clobazam VPA, valproic acid; VGB, vigabatrin.}^1\text{Based on Blumcke et al. [29–31].}
from mice that overexpressed a constitutively active form of GSK3β (GSK3β[S9A]) in the brain. Consequently, there was no difference, compared to wildtype littermates, in status epilepticus induced in these mice by an intrahippocampal injection of KA. However, we provided evidence that GSK3β attenuated epileptogenesis that was triggered by such KA treatment. GSK3β[S9A] mice also expressed higher levels of hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) and exhibited a decrease in the phosphorylation of the AMPAR GluA1 subunit at S831. In brain tissue from TLE and FCD II and III patients, the correlation was demonstrated between GSK3β phosphorylation at S9 and GluA1 phosphorylation at S845.

2. Materials and methods

2.1. Antibodies and reagents for pharmacological treatments

The antibodies that were used for this study are listed in Table 1. IRDye-conjugated secondary antibodies (IRDye 680LT donkey anti-rabbit antibody, catalog no. 926–68,023; IRDye 800CW donkey antimouse antibody, catalog no. 926–32,212) were obtained from LI-COR Biosciences (Lincoln, NE, USA). Secondary antibodies were used in dilutions that were recommended by the manufacturers. The following reagents were used for the pharmacological treatments: dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and KA (Tocris Biosciences, Bristol, UK).

2.2. Animals

All experiments described below were performed in accordance with the European Communities Council Directive of September 22nd, 2010 and Polish Act on the Protection of Animals Used for Scientific or Educational Purposes of January 15th 2015.

GSK3β[S9A] transgenic mice have been described and characterized extensively [23–25]. These mice carry a constitutively active form of GSK3β, with a mutation of S9 to A9, with the transgene under control of the mouse Thy-1 gene promoter for neuron-specific expression. Heterozygous GSK3β[S9A] mice were maintained on the FVB/N genetic background and compared to wildtype littermates in the same genetic background.

2.3. Electrophysiology in acute slices

All of the experiments were performed on 150 hippocampal slices that were obtained from 13 mice (bodyweight 20–22 g). Seventy-one and 79 slices were obtained from six wildtype and seven GSK3β[S9A] mice, respectively. Each animal was anesthetized with halothane and decapitated. The brain was rapidly removed and placed in cold (3°C) and oxygenated (95% O2 + 5% CO2) artificial cerebrospinal fluid (aCSF) that contained 121 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.25 mM KH2PO4, 1.3 mM MgSO4, 26 mM NaHCO3, and 10 mM glucose. aCSF was prepared fresh before each experiment using prefiltered and deionized water. Transverse hippocampal slices (500 μm) were obtained from both hippocampi using a tissue slicer (Stoelting, Wood Dale, IL, USA). The hippocampal slices were preincubated in oxygenated aCSF at −20°C for 45 min after dissection. After this time, the slices were transferred to a gas-liquid interface recording chamber and maintained on nylon mesh where they were continuously perfused with oxygenated and prewarmed (35°C) aCSF at a low flow rate (1 ml/min) for 45 min. Epilepticiform discharges were evoked in hippocampal slices that were obtained from each group of animals (wildtype vs. GSK3β[S9A]) by the application of KA at 0.05 and 0.5 μM. Recordings of local field potentials were performed using glass recording electrodes (3–5 MΩ, Kwik-Fil capillaries, WP Instruments, Sarasota, FL, USA). All of the recordings were performed from the CA3c region of the hippocampus, which is known to be a principal generator of field oscillations with the highest amplitude [26]. The electrodes were positioned using a motorized micropositioner (IVM-1000, Scientifica, Uckfield, UK). In all experiments, field potentials were recorded relative to ground. The signals were filtered (0.001–0.3 kHz, band pass) and amplified (1000 ×) using a P-511 preamplifier (Grass-Astromed, West Warwick, RI, USA). Micro-encephalographic (EEG) activity was displayed on a digital storage oscilloscope (Tektronix TDS 3014, Beaverton, OR, USA) and stored on a computer hard drive using a CED-1401 data acquisition interface (Cambridge Electronic Design, Cambridge, UK). The computer analysis

Fig. 2. Active GSK3β does not alter epileptiform activity in acute hippocampal slices. (a) Analogue examples of epileptiform activity that was recorded 30 min after kainic acid (KA) administration in the CA3c region of hippocampal slices from wildtype and GSK3β[S9A] mice. Epileptiform discharges were induced in hippocampal slices from each group of animals by the application of 0.05 μM KA (wildtype, n = 6; GSK3β[S9A], n = 7) and 0.5 μM KA (wildtype, n = 6; GSK3β[S9A], n = 5). Calibration: 1 s and 200 μV. (b) Quantification of the effect of bath application of KA on the frequency of epileptiform field activity in hippocampal formation slices from wildtype and GSK3β[S9A] mice. Error bars indicate SEM. (one tailed t-test).
was performed in 5 min fragments of EEG activity that was recorded 15–60 min after KA administration. Offline spectral analysis (FFT) of those fragments was performed using Spike 2.7 software (Cambridge Electronic Design, Cambridge, UK). The detailed analysis of the frequency of epileptiform discharges covered three 20 s fragments (technical repetitions) starting in the 80, 150, 220 s of the recording, respectively, that were selected from each 5 min recording.

2.4. Surgery and video-electroencephalography

Surgery, KA injections, and video-EEG were performed according to a protocol that was approved by the 1st Ethical Committee in Warsaw, Poland (decision no. 390/2017), which is in compliance with the European Community Council Directive (2010/63/EU). For KA injections and electrode implantation, the animals were deeply anesthetized with a mixture of domitor and ketamine (0.5 g/kg and 0.8 g/kg body weight, respectively, intraperitoneal). The mice were injected with 70 nl of a 20 mM solution of KA in 0.9% NaCl at a flow rate of 50 nl/min in the left CA1 field of the hippocampus as described previously [27]. During surgery, the bipolar hippocampal electrode (Bilaney Consultants, Düsseldorf, Germany) was implanted in the injected hippocampus. Cortical recording electrodes (Bilaney Consultants) were placed bilaterally in the skull over the frontal cortex, and two electrodes (one as a reference electrode and one as a ground electrode) were

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Fig. 3. GSK3 inhibitory phosphorylation correlates with GluA1 (S845) phosphorylation in temporal lobe epilepsy and focal cortical dysplasia samples. (a,b) Western blot was used to determine the levels P-GluA1 (S845), P-GluA1 (S831), GluA1, P-GSK3α (S21), P-GSK3β (S9), GSK3α and GSK3β in samples from patients with temporal lobe epilepsy with hippocampal sclerosis (TLE-HS) and in clinical malformations of cortical development (FCD and hippocampi adjacent to FCD). Tubulin is shown as a loading control. (c) Correlation analysis of P-GluA1 (S831)/GluA1 or P-GluA1 (S845)/GluA1 and P-GSK3β (S9)/GSK3β or P-GSK3α (S21)/GSK3α. The data were analyzed as Pearson and Spearman correlation coefficients (r).
placed over the cerebellum. After surgery, the mice were injected subcutaneously with antisedan (1 g/kg body weight) and connected immediately to a digital acquisition system (TWin Clinical Software for EEG, Grass Technologies, West Warwick, RI, USA). The video-EEG activity of freely behaving animals was monitored for 21 days in an isolated room. The occurrence of seizures was evaluated by visual inspection of the EEG and video recordings by trained observers. The severity of status epilepticus was analyzed during the first 24 h after the intrahippocampal injection of KA. Spontaneous seizures were defined as seizures that appeared as early as 24 h after KA administration. Seizure severity was estimated based on modified Racine’s scale [28]. Both male and female mice were used for the recordings (spontaneous seizures: n = 7 wildtype mice, n = 7 GSK3β[S9A] mice, ≥3 months old). All of the experiments were performed by experimenters who were blinded to the group assignment.

2.7. Statistical analysis

The statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA). The data were analyzed using the unpaired t-test, two-way ANOVA with Bonferroni correction, or Pearson coefficient. The exceptions were HCN4, which belongs to the HCN family, known to regulate neuronal excitability [32], and glutamate decarboxylase 1 (GAD67), a key enzyme in GABA synthesis. Their expression was increased and decreased, respectively, in the hippocampus of GSK3β[S9A] mice compared to wildtype littermates.

Previous research suggests that both the overall expression and the posttranscriptional modifications of GluA1 should be verified. For example, GluA1 phosphorylation at S831 and S845 is considered activating in animal models of seizures [5]. Therefore, we compared the levels of phosphorylated GluA1 at S831 (P-GluA1[S831]) and S845 (P-GluA1[S845]) in GSK3β[S9A] and wildtype mouse brains. The level of P-GluA1(S831) was significantly lower in GSK3β[S9A] mice, whereas P-GluA1(S845) levels were comparable in both genotypes (Fig. 1a, b). These results indicate that the chronic increase in GSK3β activity altered the expression and properties of selected ion channels, known to control neuronal excitability.

3. Results

3.1. Chronically increased neuronal GSK3β activity alters expression of HCN4 and phosphorylation of GluA1

Previous research did not provide a clear answer if GSK3β is involved in epileptogenesis. At the same time, it was shown in neuronal cultures, that GSK3β impacts expression or phosphorylation of proteins involved in synaptic transmission [14,15]. Thus, we first investigated biochemically the expression of selected proteins in the hippocampus of GSK3β[S9A] mice by western blotting with fluorescently labeled secondary antibodies (LI-COR Odyssey Imaging System). We selected proteins that are implicated in epilepsy and control synaptic transmission: ion channels, glutamate transporters, enzymes of GABA synthesis, trophic factor receptors, and various membrane glycoproteins and receptors. The expression of the majority of the tested proteins, i.e. GluA1, AMPA receptor subunit 2 (GluA2), N-methyl-D-aspartate receptor subunit NR1 (GluN1), excitatory amino acid transporter 2 (EAAT2), receptor tyrosine-protein kinase ErbB4 and tropomyosin receptor kinase B (TrkB) did not differ significantly between both genotypes (Fig. 1a, b). The exceptions were HCN4, which belongs to the HCN family, known to regulate neuronal excitability [32], and glutamate decarboxylase 1 (GAD67), a key enzyme in GABA synthesis. Their expression was increased and decreased, respectively, in the hippocampus of GSK3β[S9A] mice compared to wildtype littermates.

Based on our previous observations and available literature, we hypothesized that GSK3β increases the threshold of epileptiform discharges in response to proconvulsants. We performed a frequency analysis of epileptiform discharges that were generated in acute slices of the hippocampal formation from control and GSK3β[S9A] mice 15–60 min after KA administration (0.05 and 0.5 μM). The frequency of induced discharges is a measure of the severity of epileptiform activity [33,34].

When perfused with 0.05 μM KA, 24 of 36 (66%) of acute hippocampal slices from wildtype mice responded with epileptiform discharges. When perfused with the higher dose of KA (0.5 μM), 28 of 35 (80%) hippocampal slices from wildtype mice generated epileptiform discharges. Bath perfusion of hippocampal slices from GSK3β[S9A] mice with 0.05 and 0.5 μM KA, induced epileptiform discharges in, respectively, 20 of 41 (48%) and 23 of 38 (60%) in slices from GSK3β[S9A] mice.

Fig. 4. Active GSK3β does not impact KA-induced epileptiform discharges in acute hippocampal slices
mice \((p > .1\) for 0.05 \(\mu M\) KA and \(p = .08\) for 0.5 \(\mu M\) KA, Fisher’s exact test). In the presence of KA, at 0.05 and 0.5 \(\mu M\), hippocampal slice preparations from wildtype and GSK3β[S9A] mice responded with epileptiform discharges within 15–20 min (Fig. 2a). When perfused with 0.05 \(\mu M\) KA, the 24 hippocampal slices from 6 wildtype mice responded with epileptiform discharges that were characterized by a mean frequency of 0.25 ± 0.04 Hz. Similarly, the mean frequency of epileptiform discharges that were generated in the 20 hippocampal slices from 7 GSK3β[S9A] mice that were perfused with the same dose of KA was 0.22 ± 0.03, \((p > .1\) (Fig. 2b). The mean frequency of 0.5 \(\mu M\) KA-induced epileptiform discharges was 0.71 ± 0.03 Hz in 28 hippocampal

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**Fig. 5.** Active GSK3β decreases rate of KA-induced spontaneous seizures in vivo. (a) EEG recordings from wildtype and GSK3β[S9A] mice during spontaneous seizures (Racine scale: 3). (b) Latency to first spontaneous seizures. (c) Average number of spontaneous seizures, average seizure duration, and average seizure score at the indicated times in wildtype \((n = 7)\) and GSK3β[S9A] \((n = 7)\) mice. Error bars indicate SEM. \(*p < .05\) (unpaired t-test). (d) Cumulative number of seizures until 21 days post-KA in wildtype \((n = 7)\) and GSK3β[S9A] \((n = 7)\) mice. Error bars indicate SEM. \(*p < .05\), \(* *p < .01\), \(* * *p < .001\) (two-way ANOVA significant interaction effect, \(p = .0059\), \(* *\); two-way ANOVA with Bonferroni correction). (e) Fluctuation of average number of daily seizures during the study (two-way ANOVA, genotype effect \(p < .0001\)).
slices from 6 wildtype mice. Likewise, the mean frequency of 0.5 μM KA-induced epileptiform discharges in 23 hippocampal slices from the 5 GSK3β[S9A] mice was 0.62 ± 0.05 Hz, p = .072; (Fig. 2b). Based on these results, we concluded that a chronic increase in GSK3β activity did not significantly impact epileptiform activity in hippocampal slices acutely induced by KA.

3.3. GSK3 inhibition and GluA1 phosphorylation in temporal lobe epilepsy and focal cortical dysplasia

Based on the observed changes in the levels of proteins involved in epilepsy and synaptic transmission in GSK3β[S9A] mice in comparison to wild type mice we wanted to verify if GSK3β activity have a similar effect in the human brain. To validate our data from the preclinical models we used clinical material, i.e. epileptic tissue of various origin, namely hippocampal brain samples from patients with TLE (Table 2, Fig. 3a, c) and tissue from patients with FCD II and III and the hippocampus adjacent to dysplasia (Table 2, Fig. 3b, c). The age at surgery in the adult TLE group ranged from 25 to 57 years (mean: 36.5 years; median: 33 years), and the age at seizure onset ranged from 6 to 47 years (mean: 17.5 years). The female-to-male ratio was 4/2. The predominant seizure types were focal seizures with impaired awareness (five cases) followed by focal-to-bilateral tonic-clonic seizures (two cases) and focal aware seizures (one case). In two cases, polymorphic seizures were observed. Four patients required at least two antiepileptic drugs, whereas two patients remained on monotherapy. Hippocampal histopathology indicated H5 type 1 in five cases and H5 type 2 in one case. In the pediatric FCD group, the age range at surgery was 7–16 years (mean: 9.8 years; median: 8.5 years), and the mean age at seizure onset was 5.2 years (range: 3 months–10 years). The female-to-male ratio was 4/2. The predominant seizure types in this group were focal seizures with impaired awareness (five cases), followed by focal-to-bilateral tonic-clonic seizures (one case). All but one patient required polytherapy with at least two antiepileptic drugs. The histopathological assessment revealed FCD2A in three cases, FCD2B in two cases and FCD3A in one case. Hippocampal slices that were obtained from areas adjacent to FCD exhibited changes that were secondary to the long-lasting epileptogenic process.

In the clinical material described above, we analyzed the correlation between the levels of total and phosphorylated GSK3α, phosphorylated GSK3β, and P-GluA1 (S845 and S831). We applied a correlative analysis of the same samples instead of comparing patients’ tissue to postmortem control samples because we found the poor preservation of protein phosphorylation in the latter ones (data not shown), likely resulting from delayed autopsy tissue processing. Ratios of P-GSK3β/GSK3β and P-GluA1(S845)/GluA1 but not P-GSK3β/GSK3β and P-GluA1(S831)/GluA1 were positively correlated (Fig. 3c). In the case of P-GSK3α/GSK3α no significant correlation was observed. These data confirmed that the lower activities of GSK3β were accompanied by higher GluA1 phosphorylation at S845, in human brain samples pathologically changed due to epilepsy. These data derived from clinical material are also consistent with the data obtained from the preclinical models with regard to the correlation between GSK3 activity and GluA1 properties. As we were not able to make the same analysis in the control samples we cannot assume that the correlation is the same or different in not epileptogenic tissue.

3.4. GSK3β[S9A] decreased the frequency of KA-induced spontaneous seizures in vivo

We next investigated whether the chronic increase in the activity of GSK3β restrains KA-induced status epilepticus and subsequent epileptogenesis. GSK3β[S9A] mice and their wildtype littermates were injected intra-hippocampally with KA and the epileptiform activity was monitored by video-EEG for 21 consecutive days. The analysis of recordings from the first 24 h post-KA did not reveal significant differences between genotypes in the number, the latency, the duration, or the behavioral score of KA-induced seizures (Fig. 4). No significant differences in the latency to the first spontaneous seizures (Fig. 5b), average seizure duration, or seizure severity (Fig. 5c) were observed. In contrast, from day 14 post-KA onwards, the average number of seizures per day and the cumulative number of seizures was significantly lower in the GSK3β[S9A] mice relative to wildtype mice (Fig. 5c, d). Considering all examined period, the average number of seizures per day (Fig. 5e) was significantly different between the two genotypes (two-way ANOVA, genotype effect p < .0001).

After 21 days of seizure monitoring, we biochemically analyzed protein extracts from hippocampi of wildtype and GSK3β[S9A] mice with established epilepsy for the levels of HCN4, GAD67, P-GluA1(S831), and P-GluA1(S845). HCN4 expression was higher in GSK3β[S9A] mice compared to wildtype control mice (Fig. 6). GAD67 and P-GluA1(S831)/GluA1 levels were not different between genotypes after KA injection. Likewise, the mean frequency of 0.5 μM KA-induced epileptiform discharges in 23 hippocampal slices from the 5 GSK3β[S9A] mice was 0.62 ± 0.05 Hz, p = .072; (Fig. 2b). Based on these results, we concluded that a chronic increase in GSK3β activity did not significantly impact epileptiform activity in hippocampal slices acutely induced by KA.

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the course of epileptogenesis. We demonstrated that sustained GSK3 activity is beneﬁcial for mitigating the course of epileptogenesis. Previous studies have reported increased inhibitory phosphorylation of GSK3β at S9 and of GSK3α at S21 in response to status epilepticus, as well as in brain tissue from TLE and FCD patients. However, other studies reported completely opposite ﬁndings [16–21]. Regardless of the changes in GSK3 activity that were observed in those studies, all authors agreed and acknowledged that the activity of this essential kinase can be pathogenic. Reductions of GSK3 activity in this context were explained by parallel compensatory changes that protected the nerve cells. Conversely, increased GSK3 activity could shutdown prosurvival signaling, beside or including Tau hyperphosphorylation, and regulation of cytoskeleton dynamics [16–18, 20, 25]. The ﬁrst two processes could trigger, acute or chronic neurodegeneration, respectively, whereas the latter process could entice aberrant axonal sprouting. Nonetheless, evidence of a causal link between changes in GSK3 activity and the pathogenesis of epilepsy, including neurodegeneration and aberrant plasticity, is practically nonexistent.

In the present study, the GSK3β[S9A] mice developed fewer spontaneous seizures following KA-induced status epilepticus. Our ﬁndings demonstrate that sustained GSK3β activity is beneﬁcial for mitigating the course of epileptogenesis.

We previously reported that KA evoked signiﬁcantly less apoptosis in hippocampal organotypic slices derived from GSK3β[S9A] mice compared to wildtype mice [22]. We attributed this effect to an increase in prosurvival signaling in the brain, involving the mammalian target of rapamycin complex 2-Akt pathway [22]. Research on Lafora disease indirectly suggested that GSK3 activity acts protective by preventing the formation of Lafora bodies that accumulate improper glycogen molecules and thereby lead to neuronal pathology [36]. The present study provides additional evidence, namely the control of neuronal excitability, to these beneﬁcial effects, in which GSK3β activity protects neural networks from the effects of status epilepticus and subsequent epilepsy. When this conclusion is conﬁrmed independently, the therapeutic use of GSK3 inhibitors in epilepsy [17] should be carefully revised.

4.2. GSK3β controls neuronal excitability

Neuron-speciﬁc genetic deletion of GSK3β led to rapid and severe mortality upon pentylentetrazol-induced status epilepticus [22]. This suggested a direct involvement of GSK3β in the regulation of neuronal excitability already under basal conditions. But the present study demonstrated that frequency of KA-induced epileptiform activity was not signiﬁcantly lower in acute hippocampal slices from GSK3β[S9A] mice that express higher neuronal baseline GSK3β activity. Moreover, we did not observe less severe status epilepticus induced by KA in GSK3β[S9A] mice. Yet, these mice developed a less severe epilepsy phenotype following KA-induced status epilepticus and the biochemical analysis revealed changes potentially underlying this observation. Constitutively active GSK3β in the brain decreased the activating phosphorylation of GluA1 at Ser831 and increased HCN4 expression. We further present the correlation between increased GSK3β phosphorylation at S9 with GluA1 phosphorylation at S845 in tissue from TLE and FCD patients. These data, however, do not resolve yet the issue whether these and other ion channel–related changes functionally link GSK3β to epilepsy.

AMPARs are responsible for fast synaptic transmission in response to glutamate. AMPAR subunits can be phosphorylated at different residues, including GluA1 phosphorylation at S831 and S845. These phosphorylations are believed to activate the receptor as the result in increased channel conductance and peak-open probability, respectively [37–39]. Moreover, phosphorylation of S845 is considered the biochemical equivalent of the presence of GluA1 at the plasma membrane [40, 41]. Increased GluA1 phosphorylation was previously observed in hypoxic seizures and subsequently correlated with greater sensitivity to KA-induced seizures in mice [5]. Other studies reported higher GluA1 expression in epilepsy models [42, 43]. Our present results are fully consistent with these ﬁndings. A reasonable speculation is that GSK3 alleviates the detrimental effects of KA on neuronal activity during epileptogenesis by decreasing the pool of potentially more active GluA1 at the cell surface. Thus, the decrease in GSK3 activity that was observed in brain tissue samples from TLE and FCD patients [17] can be regarded as the manifestation of a safety mechanism, i.e. shutdown, rather than a compensatory tissue response.

The physiological role of HCN4 in neurons is poorly deﬁned. One postulated function of HCN channels is to modulate the amplitude of postsynaptic potentials and downregulate postsynaptic excitatory responses [32]. Mutations in the genes that encode HCN2 and HCN4 were discovered both in patients who suffered from epilepsy and in animal models of epilepsy [44]. Mice that lacked Hcn1 exhibited enhanced neuronal excitability that led to increased seizure susceptibility [45, 46]. Although even less is known about HCN4, a recent report showed it to be expressed in the hippocampus, mainly in CA1 and dentate gyrus fast spiking interneurons [47]. One possibility is that HCN4, in these hipo- pcamal interneurons, which discharge rapidly, becomes activated by hyperpolarization and thereby stimulate them to secrete GABA. This, in turn, inhibits their target pyramidal neurons and reduces the overall level of excitation of hippocampal networks. The increased expression of HCN4 in the GSK3β[S9A] mice demonstrated in the present study, is proposed to reduce the probability of paroxysmal discharges. Consequently, both the downregulated synaptic expression of GluA1, as well as the upregulated expression of HCN4, are complementary in supporting our hypothesis that GSK3β activity, within a certain range, prevents excessive neuronal excitation following status epilepticus and could act antiepileptically. However, we cannot exclude indirect, homeostatic effects of prolonged activation of GSK3β on other signaling pathways not mentioned above. One example, could be a potential change in GSK3α activity as this enzyme is gaining increasing recognition for its role in regulation of neuronal plasticity [both, synaptic and structural] [10, 48].

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Author contribution
MU, KK, FvL and JJ designed the experiments. MU, PK-G, TK, BC, KN, BP, KK, AG, HD, BL, and TJ performed the experiments and analyzed the data. WG, KS, SJ, K Kotulska, EV and EA contributed to the selection of patients, patient material, clinical data and the data analysis. The manuscript was prepared by the L’Oréal-UNESCO for Women and Science Fellowship in Poland and EMBO Short Term Fellowship. JJ and MU were also recipients of a Foundation for Polish Science “Mistrz” Professorial Subsidy and Fellowship, respectively.

Potential conflicts of interest
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