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Abstract

The biological processes underlying epileptogenesis following a brain insult are not fully understood, but several lines of evidence suggest that hyperphosphorylation of tau may be an important factor in these processes. To provide further insight into the causal relationship between tau and epileptogenesis, this study applied amygdala kindling to either **rTg4510 mice that, concurrent with other pathologies, overexpress phosphorylated tau,** tau knockout mice, or their respective wild-type controls. Mice were electrically stimulated twice daily, 5 days per week for three weeks. EEG was recorded to measure the primary after-discharge duration, and the behavioral progression of kindling-induced seizures was assessed. rTg4510 mice (n=10) had increased primary after-discharge durations ($p<0.001$), and significantly more rapid progression of kindling ($p<0.001$), compared with wild-type mice (n=10). Tau knockout mice (n=7), however, did not differ from their wild-type counterparts (n=8) on any of the seizure outcomes. These results suggest **that Tg4510 mice are more vulnerable to epileptogenesis**, but that the presence of tau itself is not necessary for kindling epileptogenesis to occur.

Key words: rTg4510, tau, animal model, amygdala kindling, epileptogenesis

Introduction

The epilepsies comprise a common group of neurological conditions that are characterized by the occurrence of recurrent spontaneous seizures. In some cases, anti-epileptic drugs can suppress seizures in patients with epilepsy. However, there is no treatment clinically demonstrated to prevent or reverse the biological processes that mediate the conversion of a non-epileptic brain into an epileptic brain after an epileptogenic brain insult such as traumatic brain injury, referred to as epileptogenesis. This is in part due to a poor understanding of the pathophysiology that underpins epileptogenesis. The tau protein, which stabilizes microtubules in neurons, plays a key role in maintaining functional neuronal network activity[1]. However, the excessive phosphorylation of tau results in an aggregation of the protein into intracellular 'neurofibrillary tangles' that have been identified in neuropathological environments associated with acquired epilepsy, including following traumatic brain injury, focal cortical dysplasia, and in cases of drug-resistant epilepsy[2; 3]. This association suggests that tau hyperphosphorylation may be part of the pathophysiology associated with acquired forms of epilepsy, and perhaps also promote a vulnerability to developing epilepsy.

Recent evidence directly implicates the hyperphosphorylation of tau in the epileptogenic process. The expression of phosphorylated tau is increased in the brain in the amygdala kindling and post-Status Epilepticus models of epilepsy[4; 5], and in the rat fluid percussion injury model of post-traumatic epilepsy[6]. **Also, genetically modified mice over-expressing human tau with 3 mutations linked to frontotemporal dementia including P301L exhibit hyperphosphorylated tau and spontaneous seizures, as well as other pathologies[7].** Furthermore, prevention of phosphorylated tau in acquired epilepsy models via treatment with sodium selenate reduced epileptogenesis and seizure severity, and improved functional outcomes in these studies. Similarly, other studies have reported that tau knockout

mice are protected in models of genetic epilepsy and are resistant to chemically-induced seizures[1; 8]. In addition to this preclinical evidence, the incidence of epilepsy is greater in Alzheimer's disease, a neurodegenerative condition in which hyperphosphorylated tau is a pathological hallmark[9]. To further explore the role of tau in limbic epileptogenesis, this study applied the amygdala kindling model to rTg4510 mice that express the human tau P301L variant, and to tau knockout mice, and compared kindling rates to wild-type control mice: **FVB/129 (controls for rTg4510) or C57Bl6/129 (Tau KO).**

Materials and methods

Animals

We used 3-4 month old mixed sex rTg4510 transgenic mice[10] bred on a FVB/129 background and WT littermate controls, and 4-5 month female tau knockout mice bred on a mixed C57Bl6/129sv background[11], and WT controls. Mice were sourced from our colonies at the Florey Institute of Neuroscience and Mental Health, and housed at the Department of Medicine (RMH) Biological Research Facility. All experimentation was conducted with approval from the Florey Animal Ethics Committee.

Electrode implantation surgery

Mice underwent surgical implantation of stimulating and recording electrodes at ten weeks of age, as described previously[12]. Under isoflurane general anaesthesia (2%), a stainless steel stimulating bipolar electrode (Plastics One, Roanoke, VA, USA) was implanted into the left basolateral amygdala nucleus using stereotaxic guidance (1.5 mm posterior, 2.75 mm lateral from bregma, and 3.75 mm ventral from dura). **These electrodes also served as active electrodes. In addition, we implanted stainless steel screw electrodes resting on the dura at 2mm anterior to bregma and ± 2mm lateral to midline, acting as the reference electrode (ipsilateral to active bipolar) and ground electrodes (contralateral to active).**

All electrodes were fixed to the skull with dental acrylic cement, and animals were isolated after surgery to avoid experimental loss.

Amygdala kindling

Amygdala kindling commenced on day 8 post-surgery[12]. The bipolar electrode was electrically stimulated with a one second train of 1 ms biphasic square wave pulses at a frequency of 60Hz. The after discharge threshold (ADT) was determined on the first day. Then, mice were stimulated at ADT current intensity twice daily, 5 days per week for three weeks (30 stimulations in total). The EEG was recorded by Labchart 7.0 software (ADinstruments Pty Ltd, Bella Vista, NSW, Australia). The primary afterdischarge duration were measured from the EEG trace offline by a reviewer blinded to genotype. **Behavioural seizures resulting from each stimulation were video-taped, and scored by a reviewer blinded to genotype.** The behavioural progression of kindling-induced seizures was scored according to the Racine classification: Class I, facial clonus; Class II, chewing and head nodding; Class III, contralateral forelimb clonus; Class IV, rearing and bilateral forelimb clonus; Class V, rearing and loss of balance.

Western Blotting

Following kindling, brains were excised, microdissected and the ipsilateral hippocampus processed for western blotting, as previously described[4].

Statistical analyses

Based on results from our previous studies assessing amygdala kindling rates in mice, we calculated using the t-test that to detect a 20% change in kindling rates, with the study parameters set at p=0.05 (two-tailed) and beta=0.20, the estimated minimum number of animals required in each treatment group was 7. Statistical comparisons were performed using SPSS 20.0. Repeated measures ANOVA were used to assess seizure duration and severity in the kindling experiments. **Linear regression analyses were**

conducted using Spearman's correlation coefficients. Statistical significance was set at $p<0.05$. Data represent mean ±SEM.

Results

RTg4510 mice, which harbour a P301L mutation and exhibit hyperphosphorylation of tau (Fig 1A), demonstrated significantly increased rates of kindling ($F_{(1,18)}=25.72$; $p<0.0001$, Fig 1C), as well as increased primary discharge duration ($F_{(1,18)}=26.31$; $p<0.0001$, Fig 1D) compared to wild-type littermate mice. However, we found no significant difference between rTg4510 and WT mice in after-discharge threshold currents ($t_{(18)}=0.778$; $p=0.45$, data not shown), suggesting that this does not influence the inherent excitability of the brain prior to kindling. **A**ssessing sex as a co-variate did not impact these findings. **We next correlated the expression of total tau (tau 5 immunoreactivity via western blot), and phospho-tau (S369 epitope), with seizure duration, identifying significant positive correlations (phospho-tau: $r^2=0.48$; $p=0.0008$, Fig 1E; total tau: $r^2=0.82$; $p<0.0001$, Fig 1F).**

Tau KO mice (Fig 2A) did not **significantly** differ from WT with regards to kindling rates ($F_{(1,13)}=0.07$; $p=0.79$, Fig 2B), primary discharge duration ($F_{(1,13)}=0.1.69$; $p=0.22$, Fig 2C), or after-discharge threshold ($t_{(13)}=0.536$; $p=0.60$, Fig 2D). When correlating tau and phospho-tau expression to seizure duration, trends to correlations were observed **(phospho-tau: $r^2=0.39$; $p=0.13$, Fig 2E; total tau: $r^2=0.41$; $p=0.12$, Fig 2F).**

Discussion

Several lines of evidence suggest a role for tau phosphorylation the pathophysiology of the development of epilepsy (epileptogenesis) following an brain insult: tau phosphorylation is elevated in animal models of acquired epilepsy[5] and is prevalent in post-mortem brain samples from patients with chronic temporal lobe epilepsy[3], with focal cortical dysplasia and

epilepsy, and in neurodegenerative conditions associated with epilepsy[1]. In addition, pharmacologically preventing the accumulation of hyperphosphorylated tau retards epileptogenesis in several animal models[4]. Here we extend these causal associations by demonstrating a vulnerability to kindling epileptogenesis in rTg4510 mice which exhibit elevated levels of phosphorylated tau prior to epilepsy induction, **but also provide evidence to suggest that** the tau protein itself is not exclusively required for epileptogenesis to occur. This suggests that the excessive post-translational modification of tau creates a pathological environment which facilitates epileptogenesis, and supports the concept that targeting this pathology represents a viable anti-epileptogenic strategy.

Previously, a series of studies have demonstrated that tau deletion exerts protective effects in established transgenic animal models exhibiting hyperexcitability. For example, mice with deletion of ion channels Kv1.1, Kv4.2 or Nav1.1 exhibit hyperexcitability, spontaneous seizures and increased rates of early death. Remarkably, crossing these mutant mice with tau KO mice attenuates these pathological effects[13-15], counteracting the various genetic manipulations which result in these phenotypes. Also, transgenic mouse models of AD that overexpress mutant human APP show susceptibility to chemoconvulsant-induced seizures, but this is similarly normalised after double-crossing with tau KO[8]. **Furthermore, tau KO itself[1; 8], or down-regulation via antisense oligonucleotides[16], provides seizure protection against chemoconvulsants. These data suggest that tau loss can prevent aberrant network excitability induced by genetic mutation or exposure to chemoconvulsant. This appears in contrast to our work here, which indicates no change in susceptibility to electrically-induced kindling epileptogenesis in tau KO mice, although the endogenous variation in total tau expression do appear related to seizure duration. It is unclear why such disparities exist, but may be related to the models used. The biological mechanisms involved in epileptogenesis driven by amygdala kindling**

may be different to those driven throughout development by ion channel mutation, and the loss of tau may normalise such altered developmental programs, but not mitigate the functional network changes induced by kindling.

Frontotemporal dementia (FTD) is a classic 'tauopathy', and mouse models have been generated to recapitulate these disorders. For example, the FTDP-17 mouse model has been engineered to express human tau with 4 tubulin-binding repeats as well as three missense mutations which have been identified in FTD patients: P301L, G272V and R406W[7]. These mice exhibit elevated hyperphosphorylated tau, and a previous study identified consistent spontaneous convulsive as well as non-convulsive seizures in these mice. The model used in our study is similar: insertion of a transgene expressing human tau in mouse forebrain with 4 tubulin-binding repeats but with only the P301L mutation. We did not observe any spontaneous seizures or electrographic spikes in this model, which is in contrast to the FTDP-17 model, which suggests that the single point mutation (P301L) is not sufficient to cause this severe phenotype. However, we did not systemically assess this, and we only observed mice at ages younger than that reported in the FTDP-17 study, and so it is very possible that older Tg4510 mice would express spontaneous seizures. But it does show that expressing only this single point mutation (in addition to the human tau itself) is sufficient to promote susceptibility to epileptogenesis. We also show that the expression of phospho-tau significantly correlates with seizure duration. However, in addition to hyperphosphorylated tau, the rTg4510 model also exhibits other pathologies, such as reactive gliosis, neuronal loss, inflammation, mitochondrial dysfunction, as well as other post-translational modifications of tau[10]. As such, we cannot from the current studies conclude that hyperphosphorylation of tau is the specific pathology causing accelerated kindling,

despite the observed significant correlations, and further studies would be required to determine this.

Nevertheless, there exists strong motivation to explore the consequences of elevations in p-tau on epileptogenesis, and it is intriguing to consider the mechanisms which might be responsible. Elevated hyperphosphorylated tau may promote vulnerability to epileptogenesis from a loss of function of tau: hyperphosphorylation of tau results in reduced affinity of tau for microtubules, leading to loss of axonal stability[17]. However, this explanation may be unlikely, since we have shown here that the complete deletion of tau, which would also be expected to result in loss of microtubule stability, did not similarly accelerate epileptogenesis. Alternatively, epileptogenesis vulnerability could be driven by toxic gain of function: neurofibrillary tangles composed of hyperphosphorylated tau can accumulate in neurons, astrocytes or oligodendroglia, leading to axonal and synaptic dysfunction and neurodegeneration[17]. Such pathological effects would be expected to facilitate the epileptogenic process, as observed here. The extension of this is that hyperphosphorylated tau might be pharmacologically targeted to mitigate epileptogenesis. Sodium selenate, an activator of PP2A, has been shown to inhibit epileptogenesis through decreasing hyperphosphorylated tau, but without an effect on total tau levels[4; 5]. These results suggest that drugs capable of reducing phosphorylated tau could delay the progression of epilepsy and highlights a promising area of research.

Some limitations of our study deserve to be considered. Firstly, in order to confirm the biochemical effects of our genetic manipulations, we conducted western blotting for tau and phospho-tau on freshly frozen brain tissues from the mice we used for kindling. This prevented us from confirming that the electrodes were surgically implanted into the amygdala region, which requires fixation. This is important since implantation of stimulating into different brain regions can influence kindling rates.

However, in the past we have had excellent success in performing these surgeries, with less than 10% misguided, and we think it unlikely that this had significant effects on our experimental outcomes. In addition, we studied both male and female rTg4510 mice, and kindling rates can be influenced by female sex steroids. However, sex was equally balanced between both groups, and when assessing the effect of sex, we did not find any differences between male and female kindling rates. It should also be discussed that our conclusion that kindling rates are not altered in tau KO mice is based on our study which was powered to identify a 20% change in kindling rate. A relatively small sample size (n=7-8) was required, since kindling rates in mice are quite consistent in our hands. As such, we were not powered to detect differences of less than 20%, and so we cannot rule out that a larger sample size would have identified small, but significant, changes in kindling rates in this study.

In conclusion, our results demonstrate that **amygdala kindling** epileptogenesis is accelerated in human mutant tau-expressing mice, but not **significantly** influenced by tau knockout, suggesting that hyperphosphorylated tau promotes a vulnerability to epileptogenesis and could therefore represent a potential target for mitigating the risk of epilepsy development in at-risk individuals.

Conflicts of interest

None of the authors has any conflict of interest to disclose.

Ethical conduct

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

Figure legends

Figure 1. Mice exhibiting excessive levels of hyperphosphorylated tau exhibit vulnerability to kindling epileptogenesis. (A) Representative western blot of mouse brain samples confirming the hyperphosphorylation of tau at several epitopes in rTg4510 (rTg) mice, compared to wild-type mice (WT). **(B) An example of an electrographic seizure, triggered by electrical stimulation of the amygdala. The duration of the after-discharges were readily quantifiable.** (C) Progression of kindling in rTg4510 mice (n=10) was significantly accelerated compared to wild-type mice (n=10). (C) In addition, the primary after-discharge duration was significantly longer in rTg4510 mice compared to wild-type mice. (D) There was no significant difference on after-discharge threshold between wild-type and rTg4510 mice. **(E) Hippocampal expression of phospho-tau (pS369 immunoreactivity (IR)), and (F) total tau (tau 5 IR), significantly correlate with the duration of seizures resulting from the 30th stimulation.**

Figure 2. Tau deletion does not influence progression of epileptogenesis. (A) Representative western blot of mouse brain cortex samples confirming the deletion of tau in tau KO mice, compared to WT. There were no statistically significant differences between tau KO (n=7) and wild-type mice (n=8) on kindling rates (B), primary after-discharge duration (C), or afterdischarge threshold (D). **(E) Hippocampal expression of phospho-tau (pS369 immunoreactivity (IR)), and (F) total tau (tau 5 IR), do not significantly correlate with the duration of seizures resulting from the 30th stimulation.**

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