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Role of the Fyn-PKC δ Signaling in SE-induced Neuroinflammation and Epileptogenesis in Experimental Models of Temporal Lobe Epilepsy

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Abstract

Status epilepticus (SE) induces neuroinflammation and epileptogenesis, but the mechanisms are not yet fully delineated. The Fyn, a non-receptor Src family of tyrosine kinase (SFK), and its immediate downstream target, PKC δ are emerging as potential mediators of neuroinflammation. In order to first determine the role of Fyn kinase signaling in SE, we tested the efficacy of a SFK inhibitor, saracatinib (25 mg/kg, oral) in C57BL/6J mouse kainate model of acute seizures. Saracatinib pretreatment dampened SE severity and completely prevented mortality. We further utilized *fyn*^{-/-} and *fyn*^{+/-} mice (wildtype control for the *fyn*^{-/-} mice on same genetic background), and the rat kainate model, treated with saracatinib post-SE, to validate the role of Fyn/SFK in SE and epileptogenesis. We observed significant reduction in SE severity, epileptiform spikes, and electrographic non-convulsive seizures in *fyn*^{-/-} mice when compared to *fyn*^{+/-} mice. Interestingly, significant reductions in phosphorylated pSrc-416 and PKC δ (pPKC δ -507) and naïve PKC δ were observed in *fyn*^{-/-} mice as compared to *fyn*^{+/-} mice suggesting that PKC δ signaling is a downstream mediator of Fyn in SE and epileptogenesis. Notably, *fyn*^{-/-} mice also showed a reduction in key proinflammatory mediators TNF- α , IL-1 β , and iNOS mRNA expression; serum IL-6 and IL-12 levels; and nitro-oxidative stress markers such as 4-HNE, gp91^{phox}, and 3-NT in the hippocampus. Immunohistochemistry revealed a significant increase in reactive microgliosis and neurodegeneration in the hippocampus and hilus of dentate gyrus in *fyn*^{+/-} mice in contrast to *fyn*^{-/-} mice. Interestingly, we did not observe upregulation of Fyn in pyramidal neurons of the hippocampus during post-SE in *fyn*^{+/-} mice, but it was upregulated in hilar neurons of the dentate gyrus when compared to naïve control. In reactive microglia, both Fyn and PKC δ were persistently upregulated during post-SE suggesting that Fyn-PKC δ may drive neuroinflammation during epileptogenesis. Since disabling the Fyn kinase prior to SE, either by

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treating with saracatinib or *fyn* gene knockout, suppressed seizures and the subsequent epileptogenic events, we further tested whether Fyn/SFK inhibition during post-SE modifies epileptogenesis. Telemetry-implanted, SE-induced, rats were treated with saracatinib and continuously monitored for a month. At 2h post-diazepam, the saracatinib (25 mg/kg) or the vehicle was administered orally and repeated twice daily for first three days followed by a single dose/day for the next four days. The saracatinib post-treatment prevented epileptogenesis in more than 50% of the rats and significantly reduced spontaneous seizures and epileptiform spikes in the rest (one animal did not respond) when compared to the vehicle treated group, which had >24 seizures in a month. Collectively, the findings suggest that Fyn/SFK is a potential mediator of epileptogenesis and a therapeutic target to prevent/treat seizures and epileptogenesis.

Keywords

microglia; neuroinflammation; status epilepticus; epileptogenesis; video-EEG; neurodegeneration; nitro-oxidative stress

1. Introduction

Neuroinflammation and neurodegeneration are hallmarks of epileptogenesis and temporal lobe epilepsy (TLE). In human TLE, immunohistochemistry (IHC) of brain sections from temporal lobectomy patients with history of intractable seizures, from both children and adults, provide evidence for occurrence of neuroinflammation and neurodegeneration (Beach et al., 1995; Choi et al., 2009; Das et al., 2012). We have recently demonstrated a persistent reactive microgliosis and astrogliosis, and neurodegeneration at six months after the induction of *status epilepticus* (SE) in the rat kainate model of TLE (Puttachary et al., 2016b). Neuronal hyperexcitability during a seizure, and/or prior to its occurrence, is largely attributed to intrinsic factors such as localized ionic imbalance, receptors dysfunction, and impaired release and/or uptake of neurotransmitters at synapses (Steinhauser et al., 2016; Vezzani et al., 2011). These past findings led to the discovery of several new antiepileptic drugs (AED) based on their action on the ion-channels (Bialer and White, 2010; Rogawski and Loscher, 2004; Schmidt, 2009). However, the majority of the current AEDs (including 47 failed drugs in human trials) are less effective, or do not cure the disease, and some even require lifelong administration with some potential side-effects (Kwan et al., 2011; Varvel et al., 2015). This suggests the need for development of more effective drugs that target alternative pathways to prevent/treat epilepsy. This is a major unmet clinical obligation due to poor understanding of the mechanisms of epileptogenesis. Therefore, we focused our investigation to identify a novel molecular pathway and a drugable target for epileptogenesis, and possibly as a disease modifier.

Neuroinflammation is emerging as a new mechanistic target for drug development, and it also serves as a biomarker for neuroimaging in various neurodegenerative diseases (Abi-Dargham and Horga, 2016; Albrecht et al., 2016; French 2016; Gershen et al., 2015). The microglia are considered as resident macrophages and they mediate neuroinflammation and hyperexcitability in neurons (Block, 2014; Davis and Carson, 2012; Devinsky et al., 2013). The reactive glia are known to produce proinflammatory cytokines, reactive oxygen and

nitrogen species (ROS/RNS), lipid peroxidation, hippocampal neurodegeneration, reorganization of neural circuits, and hyper-synchronicity (Bertram 2013; Goldberg and Coulter, 2013; Ryan et al., 2014; Scharfman and Binder, 2013; Vezzani et al., 2011 and 2013). The microglia become reactive in response to SE insult (Avignone et al., 2008; Puttachary et al., 2016a and b). However, the mechanism of microglial activation following seizures is largely unknown. Therefore, understanding the mechanism of activation of microglia will reveal its impact on epileptogenesis.

The Fyn is a non-receptor tyrosine kinase, a member of the Src family of kinase (SFK). It is associated with both excitatory and inhibitory ion channels, and its role in pathophysiology of synaptic transmission, plasticity, neurodevelopment, and brain injury are well known (Knox and Jiang, 2015; Kojima et al., 1998; Lu et al., 1999; Nygaard et al., 2014; Salter and Kalia, 2004). The neuronal Fyn has been known to modulate both NMDA and GABA_A receptors (Kojima et al., 1998; Lu et al., 1999), and the role of Fyn in normal amygdala kindling (Cain et al., 1995) suggests its potential association with acute seizure onset. However, its role in chronic seizures are not well known. Likewise the roles of Fyn kinase and one of its downstream targets, the protein kinase C delta (PKC δ), in microglia that mediate neuroinflammation and epileptogenesis are also not well known. Their role in microglia in experimental disease models is however beginning to emerge as has been described in Parkinson's disease (PD) models (Nygaard et al., 2015; Panicker et al., 2015). Therefore, we hypothesized that the Fyn-PKC δ signaling pathway may also mediate microglial activation in seizures, and disabling the Fyn kinase will suppress epileptiform activity and seizures by dampening neuroinflammation and neurodegeneration. We tested the hypothesis in the *fyn* knockout (*fyn*^{-/-}) and wildtype control mice (*fyn*^{+/+}, not overexpressing Fyn) bred on the same genetic background, and the rat kainate model using a pharmacological inhibitor of the SFK, saracatinib, which is in phase IIa clinical trial for Alzheimer's disease (Kaufman et al., 2015; Nygaard et al., 2015). Our results demonstrate that disabling the *fyn* kinase activity reduces: the pPKC δ -507, pSrc-416, caspase-3, proinflammatory cytokines, ROS/RNS levels, reactive microgliosis and neurodegeneration, SE severity, epileptiform spikes and spontaneous electrographic non-convulsive seizure (NCS) frequencies in the mouse kainate model. In the rat kainate model, treatment with saracatinib after SE induction suppresses epileptogenesis, and significantly reduced epileptiform spikes and spontaneous convulsive seizures in some animals.

2. Methods

2.1 Animal source and ethics statement

We used young adult (8 weeks) male C57BL/6J mice from Jackson laboratory (ME, USA), young adult male Sprague Dawley rats from Charles River (MA, USA) and *fyn*^{+/+} and *fyn*^{-/-} mice (8 weeks) for our experiments. The *fyn*^{+/+} and *fyn*^{-/-} mice were bred on C57BL/6J and Balb-c genetic background in Dr. Kanthasamy's animal breeding facility, Laboratory Animal Resources (LAR), Iowa State University (ISU). The *fyn*^{+/+} mice were used as control for *fyn*^{-/-} mice. The *fyn*^{+/+} mice neither overexpress Fyn nor contain constitutively active Fyn. The *fyn*^{-/-} mice were originally obtained from Dr. Dorit Ron's laboratory (by Dr. Kanthasamy) at the University of California, San Francisco, and are now

available from the Jackson Laboratory (stock #002271). The *fyn*^{-/-} mice were genotyped routinely by qRT-PCR to confirm *fyn* knockout. The *fyn*^{-/-} mice used in this study had normal phenotype and no obvious differences were observed in their sexual and exploratory behaviors, body weight, brain structure (gross weight and histology) when compared to the wildtype control mice (*fyn*^{+/+} mice). All animals were maintained at the LAR at ISU under controlled environmental conditions (19°C–23°C, 12 h light: 12 h dark), with *ad libitum* access to food and water. Animals purchased from Jackson laboratory and Charles River were used for experiments after four days of quarantine. All experiments were carried out in accordance with Institutional Animal Care and Use Committee (IACUC), ISU, USA (protocol number 10-12-7446/8090-MR). All surgical procedures were carried out in sterile and aseptic conditions under gaseous isoflurane anesthesia. The pre- and post-operative care were given to all animals to minimize pain and discomfort during and after surgery. Animals were monitored and weighed daily after surgery till they were used in the experiments. At the end of each experiment, the animals were euthanized by intraperitoneal (i.p.) administration of 100 mg/kg pentobarbital sodium as per the recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia of Animals.

2.2 Chemicals and reagents

Kainate (Tocris) was prepared in sterile water at the concentration of 2 mg/mL. It was always prepared fresh prior to its use. Saracatinib was purchased from Selleck Chemicals, PA, USA, and was prepared fresh in 0.5% (w/v) hydroxypropyl methylcellulose (CMC) and 0.1% tween 80 (Sigma Aldrich, USA) at the concentration of 5 mg/mL. After CMC is evenly dispersed in sterile water at 90°C, the temperature of the solution was decreased by adding cold sterile water and stored at 20°C to prevent it from precipitating at higher temperature. For perfusion, we used 4% paraformaldehyde (PFA) solution (Acros Organics, USA) in 0.1M phosphate buffer saline (PBS) at pH 7.2. The primary antibodies used in the study and their concentrations are as follows: Fyn [mouse monoclonal, 1:300 for immunohistochemistry (IHC) and 1:800 for Western blot (WB)], PKCδ (rabbit polyclonal, 1:300 for IHC 1:1000 for WB), pPKCδ-507 (goat polyclonal, 1:1000 for WB) and lamin-B (goat polyclonal; 1:500 for WB) were all purchased from Santa Cruz, CA, USA; pSrc-416 (rabbit polyclonal; 1:1000 for WB) and caspase-3 and cleaved caspase-3 (rabbit polyclonal, 1:1000 for WB) were purchased from Cell Signaling, MA, USA; 4-HNE (rabbit polyclonal, 1:300 for IHC and 1:1000 for WB), gp91^{phox} (rabbit polyclonal, 1:400 for IHC and 1:1000 for WB); 3-NT (mouse monoclonal, 1:1400 for WB), IBA1 (goat polyclonal, 1:500 for IHC) and β-actin (rabbit polyclonal, 1:10,000 for WB) were purchased from Abcam, MA, USA; NeuN (rabbit polyclonal, 1:400 for IHC, EMD Millipore, USA); Fluoro-Jade B was purchased from Histochem Inc., Jefferson, AR, USA. The secondary antibodies used for IHC were tagged with either a fluorescent dye (CY3 conjugated 1:200 or FITC conjugated 1:80) or biotin (1:500). They were purchased from Jackson ImmunoResearch laboratories, PA, USA. All the primary and secondary antibodies were prepared in 2.5% donkey (neutral species) serum to prevent cross reactivity, 0.25% sodium azide and 0.1% triton in 0.1M PBS. Streptavidin conjugates (reacts with Biotin-SP-conjugated antibodies and Biotin-SP-conjugated ChromePure proteins) were diluted in PBS alone. For WB, IRDye 680LT and 800CW donkey anti-goat or anti-mouse or anti-rabbit secondary antibodies were used at the dilution of 1:10,000. They were purchased from LI-COR Biosciences, NE, USA. The

Bradford protein assay kit was purchased from Biorad, CA, USA. For qRT-PCR, high capacity cDNA reverse transcription kit was purchased from Thermo Fisher Scientific, MA, USA; SYBR Green Master Mix was purchased from Applied Biosystems, CA, USA and the QuantiTect primer assays were purchased from Qiagen, CA, USA. We used bead-based multiplex assay (Milliplex mouse cytokine kit from Millipore MA, USA) to determine serum cytokine levels. Analytes such as anti-mouse IL-6 functional grade biotin (Cat. No. 36-7062-85) and anti-mouse IL-12 biotin (Cat. No. 13-7123-85) were purchased from Affymetrix eBioscience, CA, USA. Appropriate neutralizing IgGs for all primary antibodies were purchased from the same source as the primary antibodies.

2.3 Experimental groups and drug treatment

We used 150 mice and 14 rats in this study. The animals were randomly chosen for experimental and control groups. The first group had 30 C57BL/6J mice for saracatinib experiment. In this experiment, 15 mice were treated with saracatinib (25 mg/kg, oral gavage as a single dose) at 4h prior to a single high dose (SHD) of kainate (25 mg/kg, i. p), while the remaining 15 mice were given the vehicle [0.5% hydroxypropyl methylcellulose in 0.1% tween 80] 4h prior to kainate. The second group of 20 mice (n=10 each of *fyn*^{+/+} and *fyn*^{-/-} mice) were tested for effectiveness of SHD method of kainate (25 mg/kg, i.p) administration to induce SE. We observed 80% mortality in the *fyn*^{+/+} and 40% in the *fyn*^{-/-} mice with SHD of kainate, therefore for the rest of the experiments we followed a repeated low dose (RLD) method of SE induction with kainate (5 mg/kg, i.p., given at 30 min intervals until they showed convulsive seizures) as described previously (Puttachary et al., 2015b; Tse et al., 2014). The third group of mice (n=6 each, *fyn*^{+/+} and *fyn*^{-/-}) were used for video-EEG telemetry experiments. In this group, SE was induced 10 days after the transmitter implant after they animals recovered bodyweight. During the 10 day period of post-surgery, the baseline EEG was recorded continuously to cover both day and night cycles. The EEG recording during this period was used to evaluate the impact of surgery on spontaneous epileptiform spiking activity or seizures. The telemetry device has a built-in temperature monitoring module which gives information about their body temperature and its impact on EEG outcome. The fourth group had 88 animals (n=12 each from *fyn*^{+/+} and *fyn*^{-/-} per time-point for kainate, and 8 animals each from *fyn*^{+/+} and *fyn*^{-/-} without kainate served as naïve control for all time-points). Animals were administered with a RLD of kainate and euthanized at 4h, 24h, and 7d time-points. The last group of animals consisted of 14 rats implanted with telemetry device 10 days prior to the induction of SE, with RLD of kainate, as described previously (Puttachary et al., 2016b). In all kainate-treated animals, the behavior seizures were terminated with diazepam (10 mg/kg, i.p.) at 2h after the first onset of convulsive seizure. The 2h duration between the onset of convulsive seizure and the diazepam treatment was considered as 2h established SE. During this period, all wildtype control mice and rats had continuous convulsive seizures for >30 min. All kainate-treated animals received 1 mL of Ringer's lactate solution (s.c.) twice a day for three days to enable them to recover from dehydration and the lost bodyweight due to SE. Out of 14 telemetry implanted rats, 7 rats served as vehicle control and the other 7 rats were treated with saracatinib (25 mg/kg). The saracatinib was administered orally starting at 2h post-diazepam and repeated twice daily for first three days followed by a single dose/day for next four days during the first week of post-SE period. The rats were subjected to continuous video-EEG

monitoring for a month to quantify the impact of saracatinib post-treatment on epileptiform spikes and spontaneous seizures.

2.4 SE quantification

All animals administered with kainate were subjected to video recording. The seizures were staged and the SE was scored by direct observation of animals during the 2h SE and exact duration of each stage of seizure was calculated. The video recordings were used for secondary analysis and for independent verification of seizures by two different personnel who were not involved in direct scoring during the experiments. The personnel who did the behavioral analysis were blind to the experimental groups. The behavioral seizures were scored based on modified Racine scale from stage 1 to 5 as described previously (Beamer et al., 2012; Puttachary et al., 2015b; Racine, 1972; Tse et al., 2014). Briefly, the staging was as follows: stage 1, absence-like immobility; stage-2, hunching with facial or manual automatisms evident from brisk movement of vibrissae and repeated grooming of the face; stage 3, rearing with forelimb clonus and facial or manual automatisms; stage 4, repeated rearing with continuous forelimb clonus and falling; and stage-5, generalized tonic clonic convulsions with lateral recumbence or jumping and/or wild running followed by generalized convulsions. Stage 1 and stage 2 were categorized as non-convulsive seizures (NCS) and stage 3 as convulsive seizures (CS) (Beamer et al., 2012; Puttachary et al., 2015b; Tse et al., 2014). The latency to the onset of CS and the duration of seizures were analyzed for each animal within a group as described previously (Puttachary et al., 2015b; Tse et al., 2014). The duration of CS is the total time spent by an animal in stage 3 during the 2h of established SE until the diazepam was administered. Since a very few animals reached stage 5 in the saracatinib pretreated group and in the *fyn*^{-/-} mice, we considered stage 3 as the starting point for 2h established SE. The data were analyzed and the severity of seizures, duration of CS, latency to CS onset, and mortality rate were compared between the groups.

2.5 Procedure for transmitter device implantation and video-EEG recording

The *fyn*^{+/+} and *fyn*^{-/-} mice (n=6 each) and rats (n=7 each) were implanted with the ETA-F20 (for mice) or CTA-F40 (for rats) PhysioTel™ telemetry device (Data Science International, Minneapolis, USA) for video-EEG recording. The animals received analgesic buprenorphine (0.3 mg/kg, s.c.) prior to surgery. The animals were anesthetized with gaseous isoflurane and the mid-dorsal aspect of the head and neck was shaved and chlorhexidine scrub followed by 70% ethanol were applied using a Q-tip. The eyes were lubricated with artificial tears ointment during surgery. The surgery was performed under sterile condition while the animal was placed on a heating mat. A mid-dorsal incision was made on the head extending from just above the mid-point of the eyes to the middle of the neck. The connective tissue was separated between the skin and muscles and a subcutaneous pocket was created along the spine and flank region, which was irrigated with sterile saline before the transmitter was inserted subcutaneously. The frontalis muscle was scraped off from the bone and bilateral burr holes were drilled (2.5 mm caudal to the bregma and 2 mm lateral to the midline) without damaging the dura matter. Insulation from the tips of the electrode wires was removed, and the exposed electrodes were bent into a “V” shape and inserted into the holes to rest on the surface of the dura matter over each cerebral

hemispheres. The wires were secured in place with dental cement (mixed with methyl methacrylate liquid compound, A-M systems, WA, USA), and the exposed electrodes were completely covered with dental cement to avoid them contacting the surrounding tissues. The incision was closed with sterile surgical clips. The triple antibiotic ointment, Vetropolycin, was applied, antibiotic Baytril (Bayer pharma, PA, 5 mg/kg, s.c.) and 1 mL of dextrose normal saline were administered subcutaneously after the procedure. The nails were trimmed to prevent skin laceration and removal of clips. The telemetry device implanted animals were individually caged and placed on the PhysiolTel receivers RPC-1 connected to the data exchange matrix (specific to Dataquest A.R.T. system). The receivers detect previously matched transmitters and transmit information such as video-integrated EEG, body temperature, and activity counts from the receiver pads to the matrix and finally to the PC.

2.6 Quantification of epileptiform spikes, spontaneous electrographic NCS, and spontaneous CS

About 10 days of baseline EEG was recorded from each animal, prior to kainate treatment, which was used to normalize the EEG from post-SE period and to detect spontaneous spiking activity after surgery. The artifacts (electrical noise, exploratory behavior, grooming) were identified and excluded from epileptiform spike rate analysis. The epileptiform spikes were distinguished from artifacts based on the amplitude, duration of spikes and inter spike interval, as described in our previous publications on the mouse and rat models (Puttachary et al., 2015b and 2016b; Tse et al., 2014). After filtering the artifacts, the raw EEG was divided into 10s epochs for fast-fourier transformation (FFT) to generate power bands. The epileptiform spikes that we quantified included the spikes from the spike trains and the NCS. The spike train consisted of both spike clusters and individual epileptiform spikes including isolated pre-ictal and inter-ictal spikes. The spike clusters (<12s) included the epileptiform spikes of various amplitudes above the baseline. The electrographic NCS (>12s), associated with increased theta and delta power were quantified separately and compared between the groups. The spontaneous NCS and CS were identified and verified against real-time video and power spectrum and quantified as described in our previous publications (Puttachary et al., 2015b and 2016b; Tse et al., 2014). The identified seizures were subjected to a secondary validation by two independent observers. The number of seizure episodes and the spike rate were quantified using NeuroScore 3.2.0 software and were expressed as standard error mean (SEM) values.

2.7 LC-MS for detection of saracatinib from the hippocampus

2.7.1 Tissue processing and chemical extraction—Based on the published literature, we selected the optimum dose of saracatinib for our experiment (Green et al., 2009; Hannon et al., 2010; Liu et al., 2012; Yang et al., 2010). However, to confirm whether it crossed the BBB and whether its levels persisted for a reasonable amount of time after the drug administration, we tested the brain concentrations of saracatinib at 8h using liquid chromatography -mass spectrometry (LC-MS). The mice hippocampal tissues were homogenized in ~0.2 mL 1:1 methanol: water using bullet blender. An additional 125 µL HPLC grade water was added to the homogenized samples. This was followed by three similar successive extractions with 375 µL 3:1 acetonitrile: water. The homogenate was

sonicated for 10 min in a sonication water bath. The sample was pelleted by centrifugation at 13,000 g for 7 min and the supernatant was collected after each extraction and the samples were pooled. The pooled supernatant was dried under a dry nitrogen gas stream. The dried sample was then re-suspended in 200 μ L of 5% acetonitrile in water, filtered through a 0.2 micron PTFE syringe filter, and then subjected to LC-MS analysis.

2.7.2 LC-MS and data analysis—The LC-MS analysis for saracatinib was conducted using an Agilent Technologies 1290 Infinity Binary Pump UHPLC system coupled to an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF mass spectrometer in high resolution mode (4Gz) and scanning m/z 100–1700. LC separations were performed with Agilent Technologies Eclipse C18 1.8 μ 2.1mm X 50mm analytical column using an 18 min gradient from 100% buffer A (0.1% formic acid in HPLC grade water with 1% HPLC grade acetonitrile) to 100% buffer B (0.1% formic acid in HPLC grade acetonitrile with 1% HPLC grade water) followed by a 2 min hold in 100% buffer B and a 3 min equilibration in 100% buffer A. Saracatinib was detected as $M+H^+$ ions while using electrospray ionization in positive mode. Saracatinib detection is represented by extracted ion chromatogram (EIC) using a 10ppm extraction window and was analyzed (Fig. 1E) using Agilent Mass Hunter Qualitative Analysis B.07.00 software. The X-axis on the EIC represents retention time and the Y-axis represents the abundance of ions detected. Saracatinib concentrations were observed as the area of the EIC peaks and was calculated based on the linear curve of the observed peak areas of standards. The standard concentrations ranged from 10 femtograms/mL to 1 micrograms/mL.

2.8 Tissue processing, immunohistochemistry, imaging, and cell quantification

Animals in the telemetry group were euthanized at the end of 4 weeks and all other animals were euthanized at 4h, 24h and 7d post-SE. The brains were isolated and processed for IHC, WB, and qRT-PCR studies. The serum samples were used for cytokine assay. Serum was collected using the standard ‘serum preparation protocol’ from ThermoFischer Scientific. All animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg, i.p.). For IHC, mice were transcardially perfused with 4% PFA in 0.1M PBS (4% PFA in 0.1M PBS) under terminal anesthesia. The tissues were collected and post-fixed in the same solution for 4h at 4°C. After 4h, they were cryopreserved in 25% sucrose solution for 3–4 days at 4°C (Cosgrave et al., 2008). The tissues were then embedded in gelatin (15% type A gelatin, 7.5% sucrose, and 0.1% sodium azide in PBS, Sigma, MO, USA), wrapped in the cling film, and stored overnight at 4°C. The gelatin-embedded tissue blocks were prepared by snap-freezing in the liquid nitrogen, using iso-pentane, and were then stored in –20°C prior to cryosectioning (Beamer et al., 2012; Cosgrave et al., 2008). The coronal brain sections, 15 μ m thickness, from the tissue block were cut using CryoStar NX70 cryostat (specimen head temperature –20°C; blade temperature –16°C; trim section thickness 30 μ m; Thermo Scientific, MA, USA). The sections were collected on chrome alum gelatin (Pfaltz and Bauer, CT, USA) coated slides. The details of brain section sampling method, to represent different regions of the hippocampus (rostral to caudal) on a slide, has been described in our previous publication (Puttachary et al., 2016a). The sections were then either processed for IHC immediately or were stored in –20°C for later use. Prior to IHC, antigen retrieval was performed on the brain sections using citrate buffer (10mM citric acid, 0.05% tween 20, pH

6.0) for 20 min at 95–100°C. The sections were washed with 0.1M PBS for an hour at room temperature (RT) followed by incubation with 10% donkey serum in PBS. The sections were then incubated with primary antibodies of interest (Fyn, PKC δ , 4-HNE, gp91^{phox} and IBA1) overnight at 4°C (48h for NeuN). All new batch of primary antibodies were titrated to determine the optimum concentration. In addition to neutralizing IgG antibodies against primaries, primary antibody omission step was run as a negative control. Next day, the sections were washed with PBS for an hour at RT and probed with appropriate secondary antibodies (FITC or CY3 conjugated, or biotinylated), for an hour, at RT followed by subsequent washing with PBS and then treated with streptavidin CY3 (only biotinylated ones) for an hour. The sections were washed in PBS, and finally with water to remove salt crystals and coverslipped with vectashield containing 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. To observe the extent of neurodegeneration in the hippocampus, we did FJB-NeuN double staining. The brain sections were first stained with NeuN followed by FJB staining as described earlier (Puttachary et al., 2016b; Rao et al., 2006; Todorovic et al., 2012). For FJB staining, the sections were incubated in 0.006% potassium permanganate solution for 5–10 min with slow shaking. They were thoroughly washed twice with distilled water for a minute. The slides were submerged in 0.0003% FJB-0.1% acetic acid solution for 10 min in dark followed by 3 washes for one minute each. The slides were air-dried in the dark at RT, cleared with xylene and then mounted with surgipath acrytol (Surgipath, Leica Biosystems, IL).

For imaging, we used Axiovert 200M Zeiss inverted fluorescence microscope equipped with Hamamatsu camera (Zeiss, Deutschland, Germany). Images were captured using HCImage live 4 software (Hamamatsu Corporation, Sewickley, PA). The software has the capability to measure a large number of parameters related to size, shape, intensity, and position. All the images were taken at 20X magnification at 2s exposure. Image J was used to quantify the cells from a known area (in square microns) (Schneider et al., 2012). Bilateral cell counts were done from minimum of 4 sections per animal as described in our previous publications (Beamer et al., 2012; Cosgrave et al., 2008, 2010a and 2010b; Puttachary et al., 2016a and 2016b). The areas of cell counting for all sections on a slide and from all the groups were kept constant. The NeuN positive cells with FJB staining were counted to determine neurodegeneration. IBA1 positive cells with Fyn/PKC δ in the nucleus and/or cytoplasm alone were quantified to determine microgliosis. The DAPI staining was used to mark nuclei. Since there is no consensus on reliable and reproducible markers for reactive microglia, we considered the area of cell body, the number of branches, and the junctions to distinguish reactive microglia (also referred by some as M1-type microglia) from the resting or alternative type microglia (often referred by some as M2-type) (Andersson et al., 1991; Block, 2014; Torres-Platas et al., 2014; Walker and Lue, 2015). Initially, we derived these parameters from the resting microglia in the control brain section from a known area (e.g. CA3 region) to compare with reactive microglia derived from a similar area in the kainate treated groups at various time-points. To consider a reactive microglia as positive for Fyn or PKC δ in the cytoplasm or in the nucleus, we first set the threshold for cytoplasm and nucleus separately. The percentage of reactive microglia were derived from the total number of IBA1 positive cells. We further calculated the percentage of nuclear Fyn or PKC δ positive reactive microglia from the total Fyn/PKC δ positive reactive microglia.

2.9 Western blotting

The hippocampal tissues were dissected from both *fyn*^{+/+} and *fyn*^{-/-} animals at 4h, 24h and 7d time points immediately after euthanasia and were snap frozen in liquid nitrogen. The tissues were homogenized and lysed in RIPA buffer containing 1% protease and phosphatase inhibitors (Thermo-Scientific, USA). For cell fractionation and protein extraction from cytoplasm or nucleus, we used the kit and standard protocol from Thermo Scientific (NE-PER™ Nuclear and Cytoplasmic extraction reagents; catalog number 78833). The hippocampal tissues were homogenized in 200 ul of cytoplasmic extraction reagent I (CER I). The samples were vortexed vigorously for 15 seconds to suspend the pellet followed by incubation in ice for 10 min. After incubation, 11 ul of cytoplasmic extraction reagent II (CER II) was added followed by repeated vortexing and ice incubation steps. The samples were then centrifuged at 16,000 x g for 5 min. The supernatant (cytoplasmic extract) was collected and the pellet was re-suspended in 100 ul of ice-cold nuclear extraction reagent (NER) followed by repeated vortexing for 15 seconds and ice incubation for 10 min for the total of 40 min. The samples were then centrifuged at 16,000 x g for 10 min. The supernatant (nuclear extract) was collected and stored in -80°C for later analysis. The protein concentration from tissue lysates were determined using the Bradford assay kit (Biorad, USA). Equal amounts of protein (40 ug) was loaded in the wells of precast gels along with the molecular weight marker. The gels were run at 100V for 1–2h at RT until the bromophenol dye reached at least 0.5 cm from the bottom of the plate. The proteins were transferred onto the nitrocellulose membrane and the transfer sandwich was placed into a mini transfer blot unit (Biorad, USA) at 4°C overnight at 25V for 14h according to the manufacturer's instructions. Next day, the membrane was washed with PBS and 0.05% tween 20 (PBS-T) for 1h followed by blocking with Fluorescent Western Blot blocking buffer (to avoid non-specific binding) (Rockland Immunochemicals, PA, USA) in PBS with 0.05% tween 20 at RT. After the blocking step, the blots were washed twice for 15 min each with PBS-T and probed with primary antibodies of interest overnight at 4°C. The following day, the membrane was incubated with IR-680 or IR-800 dyes (1:10000, LiCor, USA) followed by further washes with PBS-T as described earlier. The β -actin was used as a loading control for cytosolic fractions and lamin-B for nuclear fractions. Fluorescent Western Blotting blocking buffer with 0.05% tween 20 was used as a diluent for both primary and secondary antibodies. The bands were identified using Odyssey IR imaging system and were quantified using imageJ software and normalized with the β -actin.

2.10 qRT-PCR

RNA was extracted using the trizol chloroform (ThermoFisher Scientific) extraction method as described previously (Cosgrave et al., 2008; Seo et al., 2014). One microgram of RNA was used for reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) yielding high quality single stranded cDNA. Quantitative RT-PCR was performed for the following genes using SYBR Green Mastermix (Applied Biosystems, CA, USA) with pre-validated qPCR primers. Primers for *IL-1 β* , *iNOS*, and *TNF- α* were purchased from QuantiTect Primer Assay (Qiagen, USA). The house keeping gene, 18S rRNA (Qiagen, MD, USA), was used in all qPCR experiments for normalization. No-template controls (NTCs) and dissociation curves were obtained for all experiments to exclude cross-contamination. The fold change in the mRNA expression was determined

using cycle threshold (C_t) values for the genes of interest and also for the housekeeping genes.

2.11 Multiplex cytokine immunoassays

Cytokine levels were assessed from the serum using Luminex assay kit as described previously (Panicker et al., 2015). A five-fold dilution of serum was made with 0.1M PBS containing BSA. 40 μ L of diluted serum was then added to the equal amount of primary antibodies, conjugated to magnetic microspheres, followed by overnight incubation at 4°C in a clear bottom black 96-well plate. After incubation, each well was triple-washed using a magnetic washer and then incubated for an hour with secondary antibodies followed by three secondary washes. The samples were incubated for 30 min with streptavidin/phycoerythrin followed by two additional washes. All assays were done in duplicates and previously known positive control and a negative control without primary antibodies were used simultaneously with the test samples. A Bioplex reader was used to read the 96-well plates. A standard curve of all the cytokines was prepared using standard cytokines (Peprotech).

2.12 Experimental design, methodological rigor, and statistical analyses

We had consulted a Biostatistician, Dr. Wang, College of Veterinary Medicine, Iowa State University, for experimental design and statistical analyses for this study. We chose the most appropriate control (strain of mice or the vehicle) and statistics for each set of experiments. For example, for the saracatinib experiment to determine its anti-seizure property we used C57BL/6J mouse kainate model of seizure, and for KO experiments the same genetic background for the *fyn*^{-/-} mice and the wildtype control mice were used. To determine saracatinib's anti-epileptogenic or disease modifying effects, we chose the rat kainate model and post-SE treatment regimen since pretreatment reduces SE severity and compromises epileptogenesis. In the rat kainate model, in contrast to the mouse model, occurrence of spontaneous CS are reliable and progressive (Puttachary et al., 2016b).

The experimenters were blind to the experimental groups until the data analyses were completed. Transgenic mice genotyping was done using qRT-PCR to confirm the gene knockout. We followed pre-determined criteria to exclude animals from data analyses. The criteria set were: i) if animals do not respond to the predetermined kainate dose (a maximum of six doses of 5 mg/kg in RLD method; 25 mg/kg in SHD method); ii) if animals die during the course of experiment; and, iii) in telemetry group if animals do not regain bodyweight within 8–10 days. We had taken measures to minimize variables by: i) randomizing the animals based on predetermined weight (20 g mice or 200 g rat) and age range (8 weeks) before the start of experiment; ii) seizure severity during the SE was quantified by both direct observation and offline video analysis by at least two independent observers; iii) we acquired ~260 h of baseline EEG data, covering at least 10 day-night cycles, to normalize post-SE EEG from the same animal; iv) where appropriate, we implemented the first two of the three principles of reduction, refinement, and replacement (3Rs) by adopting a refined RLD method of SE induction, which reduced mortality rate and minimized variability in SE severity between animals and groups; and, v) we determined the optimum concentration of

the primary antibodies by serial dilution and validated their specificity using neutralizing antibodies appropriate to the primary antibodies.

For statistical analyses, we compared the standard error means between the groups using the Fisher's exact test, the Mann-Whitney test, one-way ANOVA with Bonferroni multiple comparison post-test or two-way ANOVA where appropriate. For example, two-way ANOVA with appropriate degrees of freedom was used to calculate seizure frequency since they do not follow a normal distribution (Figs. 1-A, 2-D, 3-F, 3-I, 10A and 10D). Further information on statistical test/s for each figure is described in legends. We used the GraphPad Prism 5 for all statistical analysis. The p-value 0.05, at 80% power, was considered as statistically significant difference between the groups, which was the basis for determining the group size.

3. Results

3.1 The Fyn/SFK inhibitor, saracatinib significantly reduced the severity of SE and prevented mortality during SE

The saracatinib is a broad spectrum SFK inhibitor (Green et al., 2009; Hennequin et al., 2006), and is in the clinical trials for treating Alzheimer's disease (Nygaard et al., 2015). Since saracatinib also targets the hippocampus, an important ictogenic foci of the brain, we tested its impact on SE onset. Saracatinib (25 mg/kg) or the vehicle was administered orally as a single dose at 4h prior to the induction of SE with kainate. The dose was selected based on the published literature (Green et al., 2009; Hennequin et al., 2006). We tested the effect of a SHD of kainate (25 mg/kg) on behavioral seizures onset, and compared the seizure severity, duration of convulsive seizures, latency, and survival rate between the groups. The studies revealed a significant reduction in seizure severity and the duration of CS, and increased latency to the onset of first CS in the saracatinib treated mice when compared to the vehicle control group during the 2h established SE (Fig. 1A–C). There was no mortality in the saracatinib treated group due to a significant reduction in the severity of seizures. As expected, about 20% mortality occurred in the vehicle-treated control group due to severity of kainate-induced seizures (Fig. 1D). In the SHD of kainate group, 67% of the mice reached stage 5 seizure in the vehicle group, while only 7% reached stage 5 and they spent less time in CS stages in the saracatinib group (Fig. 1B) suggesting the role of Fyn and other SFK in seizure modulation. LC-MS analysis of the hippocampi confirmed that the saracatinib crossed the BBB and it persisted at high levels at 8h (223.8 ± 22.01), and were also detected at 28h post administration (2.693 ± 0.1714) ($r^2 = 0.9665$; $***p < 0.0001$) (Fig. 1E–F).

3.2 Fyn knockout reduced SE severity, mortality, epileptiform spikes, and spontaneous NCS and CS during the first 4 weeks of post-SE

Having observed a significant reduction in SE severity with saracatinib treatment, we tested further whether Fyn kinase has a role in the mechanism of seizure onset. We used *fyn*^{-/-} and the wildtype control mice for SE induction. First, similar to saracatinib experiment, we used a SHD of kainate (25 mg/kg) for both *fyn*^{-/-} and *fyn*^{+/+} mice. Interestingly, unlike the vast majority of C57BL/6J mice, the *fyn*^{+/+} hybrid mice did not show a progressive seizure

pattern from stage 1 to 5, instead the majority of them (90%) directly reached stage 5 seizures in <20 min of kainate administration, and 80% of them died during SE (Fig. 2A–B). In contrast, *fyn*^{-/-} mice showed fewer episodes of stage 5 seizures in 30% of the mice, but mortality rate was 40% (Fig. 2B) even after diazepam administration. Animals that had severe SE, during the 2h of established SE, excessive salivation was observed more frequently in *fyn*^{+/+} mice than in *fyn*^{-/-} mice. Another interesting observation was their sensitivity to diazepam treatment. Both *fyn*^{+/+} and *fyn*^{-/-} mice took longer time to recover from diazepam treatment (almost 12h) compared to C57BL/6J mice with the same dose (10 mg/kg) and they recovered in <3h after diazepam treatment.

Since there was increased mortality rate in both *fyn*^{+/+} and *fyn*^{-/-} mice with SHD of kainate, we used RLD of kainate method to reduce mortality and to understand seizure progression during SE. We pooled the SE behavioral data from all time-points in non-telemetric groups and compared between the *fyn*^{+/+} and *fyn*^{-/-} mice. Both groups received similar numbers of RLD of kainate, therefore there was no significant difference in the total amount of kainate administered between the groups to reach convulsive seizures (Fig. 2H). Also, there was no significant difference between the groups in latency to the first onset of convulsive seizure (Fig. 2F), but there was a significant reduction in the seizure severity (Fig. 2D–E). Interestingly, the mortality rate in *fyn*^{-/-} mice was higher than *fyn*^{+/+} mice (22% vs. 10%; Fig. 2G) with the RLD method of SE induction with kainate, which was unexpected. Out of 22% mortality in the *fyn*^{-/-}, 14% was after diazepam treatment.

The telemetry implanted mice were subjected to continuous (24/7) video-EEG monitoring to quantify epileptiform spikes and spontaneous seizures frequency during the first four weeks of post-SE. Prior to kainate administration, none of the mice showed epileptiform spike or seizure during the first 10d post-surgery. A representative EEG traces during the SE (Fig. 3A) and post-SE between *fyn*^{+/+} and *fyn*^{-/-} mice (Fig. 3D) are shown. Increased delta and theta powers, and decreased gamma power were correlated with the epileptiform spike characteristics on EEG in NCS (Fig. 3E). During the SE, there was about 50% reduction in the duration of CS in the *fyn*^{-/-} mice when compared to the *fyn*^{+/+} mice (Fig. 3C). Unlike the C57BL/6J mice reported in our publication (Puttachary et al., 2015b), the number of spontaneous CS were very few (average 5 episodes) in the *fyn*^{+/+} and spontaneous CS were completely absent in the *fyn*^{-/-} mice. Therefore, we quantified electrographic NCS and the epileptiform spike frequency in both groups as described previously in our publication (Puttachary et al., 2015b). We considered spike trains and the epileptiform spikes within seizure clusters (<12s duration) to determine the epileptiform spike frequency. An example of an electrographic NCS, the spike train, and spike clusters is illustrated (Fig. 3H). During the 28d post-SE, both spontaneous electrographic NCS and the epileptiform spikes frequencies were significantly reduced in *fyn*^{-/-} when compared to *fyn*^{+/+} mice (Fig. 3F–G, I–J).

3.3 SE significantly increased both Fyn and phosphorylated Src kinase levels in the hippocampus of *fyn*^{+/+} mice, but not in *fyn*^{-/-} mice

To understand the molecular mechanism of microglial activation mediated by the Fyn kinase during epileptogenesis, we tested the levels of Fyn and phosphorylated Src (pSrc-416) in the

hippocampus at 4h, 24h, and 7d post-SE. The Western blot analysis revealed a significant increase in both Fyn and pSrc-416 levels at all the time points in the hippocampus of *fyn*^{+/+} mice, except the cytosolic Fyn at 4h, when compared with naïve control (Fig. 4A–D). As expected, total Fyn levels were not detected in *fyn*^{-/-} mice, however, pSrc-416 levels were detected, but there were no significant differences between naïve control and SE groups. At 24h and 7d post-SE, the pSrc-416 levels were significantly lower in *fyn*^{-/-} mice when compared to *fyn*^{+/+} mice (Fig. 4D). This may suggest that other members of the SFK may have a minor role in epileptogenesis. Further analysis of the fractionated samples revealed a significant increase in Fyn levels in both the cytoplasmic and nuclear fractions at all the time points in *fyn*^{+/+} mice when compared to naïve control (Fig. 4A–C).

3.4 Fyn selectively upregulated in the microglia and hilar neurons of the dentate gyrus in *fyn*^{+/+} mice during post-SE

IHC of brain sections, double stained for neuron (NeuN) or microglia marker (IBA1) and Fyn, revealed a pattern of Fyn staining in neurons, and in the cytoplasm and nuclei of reactive microglia in the hippocampus and dentate gyrus (4E–J). The pyramidal neurons in CA1 and CA3 regions of the hippocampus did not show increase in Fyn staining at any time point tested during the post-SE. However, the Fyn positive neurons in the hilus of dentate gyrus were significantly increased at all the time-points when compared to the control (Fig. 4E–F).

Interestingly, we also observed an increase in the number of FJB positive neurons in the hilus at these time points in *fyn*^{+/+} mice (Fig. 9B). Moreover, a significant increase in reactive microglia was also observed at 4h, 24h, and 7d in *fyn*^{+/+} mice when compared to naïve control (Fig. 4G, I). At 24h and 7d, intense Fyn positive microglia were found in CA1 (not shown), CA3 (Fig. 4G), and the dentate gyrus (not shown). The vast majority of these microglia resembled M1-like phenotype with thick cytoplasmic process and often multinucleated with a large cell body. In these cell types, the Fyn was localized in the nucleus (Fig. 4G–H, J). In contrast, the *fyn*^{-/-} mice did not show any Fyn staining, and the vast majority of microglia had M2-like phenotype or alternatively activated type morphology with a small soma and thin cytoplasmic processes (Fig. 4G). However, there was a significant increase in reactive type microglia at 4h and 24h, and a marginal increase at 7d post-SE in the *fyn*^{-/-} mice was observed when compared with the naïve control (Fig. 4I). A similar pattern of Fyn staining in microglia was detected in other parts of the brain such as the entorhinal cortex, thalamus, and amygdala (data not shown).

3.5 SE significantly increased naïve and phosphorylated PKCδ levels in the hippocampus of *fyn*^{+/+} mice when compared to *fyn*^{-/-} mice

It was known that phosphorylated SFK (pSrc-416) activates PKCδ and translocates to the nucleus of microglia in cell culture and animal models of PD (Panicker et al., 2015; Saminathan et al., 2011). Therefore, we speculated that a similar mechanism may exist in the kainate model of epileptogenesis. In the *fyn*^{+/+} mice, the Western blot analysis of proteins from the hippocampus revealed a significant increase in both the naïve full-length PKCδ and phosphorylated PKCδ (pPKCδ-507) at 4h, 24h, and 7d post-SE when compared with the control (Fig. 5A–D). Since pSrc-416 levels were detected in *fyn*^{-/-} mice, we also

observed an increase in the levels of both full-length PKC δ and pPKC δ -507 at all time points. Further analysis of the nuclear fractions from the hippocampus, revealed a significant increase in PKC δ levels in the *fyn*^{+/+} mice, but not in the *fyn*^{-/-} mice (Fig. 5C). In concurrence with the Western blot results, IHC of the brain sections revealed a significant increase in both the numbers of PKC δ positive microglia as well as increase in intensity of staining in the nuclei of the reactive microglia in the hippocampus at 4h, 24h, and 7d time points (Fig. 5E–F) in the *fyn*^{+/+} mice. In the *fyn*^{-/-} mice, there was a marginal increase in microglial cytoplasmic PKC δ over time, but there was no significant increase in the nuclear PKC δ (Fig. 5E–F). In the hilus of dentate gyrus, we observed a transient increase in the PKC δ staining in neurons (data not shown).

3.6 SE significantly increased caspase-3 and cleaved caspase-3 levels in the hippocampus of *fyn*^{+/+} mice when compared to *fyn*^{-/-} mice

The PKC δ is cleaved by caspase-3 to cause neuronal death (Kaul et al., 2003; Kato et al., 2009; Kitazawa et al., 2005), therefore we tested the caspase-3 and cleaved caspase-3 levels in the hippocampus. There was an increase in the caspase-3 and cleaved caspase-3 levels at all time points in both *fyn*^{+/+} and *fyn*^{-/-} mice groups when compared with the respective controls (Fig. 6A–C). However, in the *fyn*^{-/-} mice, the caspase-3 and cleaved caspase-3 levels were significantly lower at 4h and 24h post-SE when compared with the *fyn*^{+/+} mice (Fig. 6A–C).

3.7 Key proinflammatory cytokines profile during epileptogenesis in *fyn*^{+/+} and *fyn*^{-/-} mice

It has been demonstrated that PKC δ translocation to the nucleus initiates transcription of proinflammatory cytokines and iNOS in M1-like microglia (Bujor et al., 2011; Gordon et al., 2016). Since we found the PKC δ translocation to the nucleus in microglia, which also had M1-like phenotype (large cell body, thick cytoplasmic process), we further investigated whether it has an effect on proinflammatory cytokines in the hippocampus and serum. We utilized quantitative RT-PCR for mRNA assay for the hippocampus and multiplex cytokine assay for the serum. We found an increase in the TNF- α , IL-1 β , and iNOS mRNA levels in the *fyn*^{+/+} mice when compared to the control at 4h, 24h, and 7d time points (Fig. 7A–C). In the *fyn*^{-/-} mice, the IL-1 β mRNA was significantly increased at 7d, and TNF- α mRNA at 24h post-SE, however iNOS mRNA expression levels did not change significantly (Fig. 7A–C). However, when compared between the groups, the TNF- α expression was significantly higher at all the time points, while the IL-1 β at 24h and the iNOS at 7d post-SE. Multiplex cytokine assay of the serum revealed a significant increase of both IL-6 and IL-12 levels at all the time points in the *fyn*^{+/+} mice when compared with the control (Fig. 7D–E). Interestingly, in the *fyn*^{-/-} mice, the IL-12 levels were upregulated at all time points, but the IL-6 levels were only marginally increased at 24h post-SE (Fig. 7D–E). When their levels were compared between the groups, IL-6 levels, but not IL-12, were significantly reduced in the *fyn*^{-/-} mice when compared to the *fyn*^{+/+} mice at 4h and 24h post-SE (Fig. 7D–E). Other cytokines mRNA or proteins levels were undetectable at all three time points.

3.8 Nitro-oxidative stress markers in the hippocampus during epileptogenesis in *fyn*^{+/+} and *fyn*^{-/-} mice

The phosphorylated PKC δ activates p47^{phox}, the cytosolic subunit of NOX2, which forms a functional complex with the membrane associated gp91^{phox} to activate NOX2 signaling pathway and drives ROS and RNS production (Bedard and Krause, 2007; Fontayne et al., 2002). In this study, we quantified nitro-oxidative stress markers; gp91^{phox}, 4-HNE, and 3-NT levels from the hippocampus by employing IHC and WB methods. The 4-HNE levels were significantly increased at 24h and 7d in the *fyn*^{+/+} mice, while gp91^{phox} and 3-NT levels were upregulated in both *fyn*^{+/+} and *fyn*^{-/-} mice at all time points when compared with their respective controls (Fig. 8A–D). All three markers were significantly decreased at 24h and 7d post-SE in the *fyn*^{-/-} mice when compared with the *fyn*^{+/+} mice. At cellular level, their expression was predominantly in the microglia (Fig. 8E), however, neurons and a few astrocytes were also immunoreactive to these markers (data not shown). When compared between the groups at 24h and 7d post-SE, we observed a large number of gp91^{phox} containing reactive microglia in the hippocampus in the *fyn*^{+/+} mice (Fig. 8E). Their numbers significantly reduced in the *fyn*^{-/-} mice (Fig. 8G). Likewise, the numbers of 4-HNE and 3-NT positive neurons were also changed between the groups in the entorhinal cortex, amygdala, and the thalamus (data not shown).

3.9 The FJB positive neurons increased in the hippocampus during epileptogenesis in both *fyn*^{+/+} and *fyn*^{-/-} mice

Having observed increased levels of nitro-oxidative stress markers, proinflammatory cytokines, cleaved caspase-3, and increased expression of Fyn and PKC δ levels in microglia of the hippocampus during epileptogenesis, we were interested to find out the extent of neurodegeneration in the hippocampus. We confirmed this by staining the brain sections with FJB and NeuN. There were significantly more numbers of degenerating neurons in the hippocampus at all time points in both groups when compared to their respective controls (Fig. 9A–B). This suggest that Fyn alone has little impact on neurodegeneration during epileptogenesis. Although the numbers of FJB positive neurons decreased at 7d post-SE in both groups, overall they were significantly lower in *fyn*^{-/-} mice at 24h post-SE in CA3 region of the hippocampus and the dentate gyrus when compared with the *fyn*^{+/+} mice (Fig. 9B).

3.10 The saracatinib post-treatment in the rat kainate model prevented or modified epileptogenesis

Disabling the Fyn kinase function, either by pretreatment with saracatinib or *fyn* KO, prior to SE induction impacts the initial severity of SE and thus compromises the epileptogenic events. Therefore, we tested the effect of Fyn kinase inhibition with the saracatinib on epileptogenesis after the induction of SE in the rat kainate model of TLE. To mimic human TLE from translational view point, the rat kainate model is more suitable in terms of progressive nature of the disease since the frequency of spontaneous CS consistently increases over time in rats in contrast to the mouse kainate model (Puttachary et al., 2015b and 2016b). Therefore, we tested the saracatinib in the rat kainate model after terminating the behavioral SE with diazepam. The saracatinib (25 mg/kg, oral) was administered at 2h

post-diazepam and repeated twice daily for first three days followed by a single dose daily for the next four days during the first week of post-SE period. The treatment regimen was chosen based on increase in Fyn and PKC δ levels during the first week of post-SE in the mouse model. Interestingly, four out of seven rats treated with the saracatinib did not develop epilepsy, and in two rats the number of spontaneous CS were <10 in a month in contrast to >24 seizure episodes in the vehicle treated group. However, one rat did not respond to saracatinib treatment (Fig. 10C). All the 7 rats treated with the vehicle became epileptic and had >24 spontaneous CS in a month long continuous video-EEG study. The number of spontaneous CS and epileptiform spike counts were also significantly reduced in the saracatinib treated rats when compared with the vehicle treated group (Fig. 10A–B, 10D–E). It is also important to note that there was no significant difference in the initial SE severity between the saracatinib and the vehicle treated groups (Fig. 10F).

4. Discussion

In the present study, we provide evidence for the role of Fyn-PKC δ signaling as a novel mechanism of microglial activation during epileptogenesis in the kainate model of TLE. The role of neuronal Fyn in synaptic transmission and plasticity, LTP, epileptiform activity, neurodevelopment, and ischemic brain injury have been well known (Kaufman et al., 2015; Kojima et al., 1998; Lu et al., 1999; Nakazawa et al., 2001; Nygaard et al., 2014). However, its role in neuroinflammation-mediated epileptogenesis are unknown. We demonstrate that the Fyn upregulation occurs in the microglia and in the neurons of the hilus of dentate gyrus, but not in the hippocampal pyramidal neurons, during epileptogenesis. For the first time we demonstrate the role of Fyn and PKC δ in reactive microglia and their impact on proinflammatory cytokines, nitro-oxidative stress biomarkers, and neurodegeneration in the mouse kainate model of TLE. Further, a continuous (24/7) video-EEG monitoring provided real-time evidence for epileptiform spiking and spontaneous seizure occurrence as functional readouts for the assessment of the lack of *fyn* gene during SE and epileptogenesis, and the impact of inhibiting Fyn function during post-SE on epileptogenesis. As a proof-of-concept for translational purpose, a pharmacological inhibitor of the SFK, saracatinib was tested in the mouse and rat models of TLE to demonstrate its anti-seizure and antiepileptogenic or disease modifying properties.

The targeted deletion of *fyn* showed resistance or slower rate of epileptogenesis (Cain et al., 1995; Kojima et al., 1998). In transgenic mice, overexpressing a constitutively active form of Fyn showed decreased seizure threshold and higher mortality (Kojima et al., 1998). In our study, a SHD of kainate (25 mg/kg) caused 80% mortality in *fyn*^{+/+} mice. These mice are normal wildtype bred on a similar genetic background as *fyn*^{-/-} mice, and they do not overexpress Fyn. The RLD method of SE induction with kainate (Tse et al., 2014) reduced mortality during the SE in both *fyn*^{+/+} and *fyn*^{-/-} groups. However, in *fyn*^{-/-} mice, mortality was higher after the diazepam administration (Fig. 2G), which could be due to abnormal GABA_A receptor activity in the absence of Fyn kinase (Boehm et al., 2004; Jurd et al., 2010; Knox and Jiang, 2015; Lu et al., 1999). It has been shown that Fyn deletion can cause abnormal GABAergic synaptic transmission resulting in behavioral, functional, and developmental abnormalities in different brain regions (Knox and Jiang, 2015). However, the extent of morphological abnormality in the hippocampus and functional deficits in the *fyn*

KO mice depend on strains/genetic background (Grant et al., 1992; Kojima et al., 1998; Lu et al., 1999). The *fyn*^{-/-} mice developed on C57BL/6J and Balb-c background, used in this study, did not show morphological changes in the hippocampus. In contrast, *fyn*^{-/-} mice bred on C57BL/6J x S129 background showed varying degree of hydrocephalus (unpublished). The increased mortality in the *fyn*^{-/-} mice in this study could also be due to the residual effects of kainate in the brain. The RLD method of kainate administration was chosen with the intention of achieving severe SE and to reduce mortality. It is important to note that the kainate levels persisted in the hippocampus for >24h (unpublished). It is likely that the first dose of kainate increases the BBB permeability (Puttachary et al., 2016b), which allows large quantities of kainate into the brain with subsequent dosing. It has been shown that RLD of kainate induces mGluR5 receptor expression in astrocytes (Umpierre et al., 2016), which could impact synaptic function. The mGluR5 is also associated with Fyn signaling (Nygaard et al., 2014), and it facilitates both ionotropic glutamate and GABA_A receptors at central synapses (Xiao et al., 2006). Therefore, the activation of the receptors in the absence of Fyn may have a different outcome such as the diazepam-induced death in this study.

The chemoconvulsant-induced SE leads to the development of epilepsy (Buckmaster, 2004; Puttachary et al., 2016b; Williams et al., 2009). The most common features of epileptogenesis are increased epileptiform spike rate, reactive gliosis, neurodegeneration, and altered synaptic plasticity (Han et al., 2016; Puttachary et al., 2016a; Rattka et al., 2013; Robel et al., 2015; Sutula, 2004; White et al., 2010). It is still unclear which of these processes starts first, and which cell types play critical role at different stages of epileptogenesis. The role of both GABA_A and NMDA receptors in the onset of acute seizures has been well known, and several AEDs have been discovered to modulate these receptors to prevent seizure onset (Bialer and White, 2010; Loscher, 2002; Rogawski and Loscher, 2004; Schmidt, 2009). These receptors activity at the postsynaptic terminal can also be modified by the signaling molecules associated with the PSD, for example, PSD-95, SFK including Fyn, neuronal NOS, tau, and several other molecules. The role of NMDAR in pyramidal neuronal excitation during SE and epileptogenesis has been well described in several experimental models of epilepsy (Dingledine et al., 1990; Gataullina et al., 2017; Ghasemi and Schachter, 2011; Moussa et al., 2001; Naylor et al., 2013; Rice and DeLorenzo, 1998). Studies have shown an increased SFK mediated tyrosine phosphorylation of NMDAR complexes after experimentally induced seizures in animals (Kojima et al., 1998; Moussa et al., 2001; Nakazawa et al., 2001; Salter and Kalia, 2004; Zheng et al., 1998). The role of neuronal Fyn in modulating GABA_A and NMDA receptor function is well known (Cain et al., 1995; Knox and Jiang, 2015; Lu et al., 1999; Kojima et al., 1998). Neuronal Fyn involvement in normal amygdala kindling has also been known for more than two decades (Cain et al., 1995). However, Fyn is not required for maintenance of kindling implying that neuronal Fyn may be limited to acute seizure onset. Our results from Fyn/SFK inhibitor, saracatinib pretreatment experiment in the mouse kainate model of acute seizures confirm that disabling Fyn/SFK prior to SE induction dampens the severity of seizures (Fig. 1A–B). IHC of brain sections from the wildtype control animals that had severe SE confirmed that Fyn was not upregulated in the excitatory pyramidal neurons of the hippocampus, but in the hilar neurons of the dentate gyrus at 4h, 24h and 7d post-SE (Fig.

4E–F). It has been known that the neurons in the hilus of dentate gyrus contain GABA, calbindin, somatostatin, neuropeptide-Y, cholecystokinin, and parvalbumin (Hofmann et al., 2016; Houser, 2007; Long et al., 2011; Marx et al., 2013; Megahed et al., 2015; Sik et al., 1997; Sun et al., 2007; Sundstrom et al., 2001). SE has been known to cause the loss of such inhibitory neurons in the hilus (Hofmann et al., 2016; Long et al., 2011; Marx et al., 2013; Pitkanen et al., 2007; Sun et al., 2007). The fluoro-jade B and NeuN co-staining confirmed a significant increase in the FJB/NeuN positive neurons in the hilus of dentate gyrus (Fig. 9B) suggesting that the loss of inhibitory neurons during post-SE partly contributes to epileptogenesis.

The role of microglia in epileptogenesis and the time of their activation following SE is intriguing. Earlier studies, based on morphology, have reported that microglia become reactive after 24–36h post-SE in animal models (Andersson et al., 1991; Avignone et al., 2008; Beamer et al., 2012; Sabilallah et al., 2016). However, the two-photon real-time brain imaging, *in vivo*, has revealed that the microglia become activated in <30 min of post-insult and they engage in pruning the dendritic spines and synaptic re-organization. Activated microglia also produce trophic factors as an early protective mechanism (Parkhurst et al., 2013; Szalay et al., 2016; Vezzani et al., 2011). The microglia are highly mobile and dynamic cells. The chemokines and fractalkines facilitate their migration to the site of hyper-excited neurons (Eyo et al., 2017; Harrison et al., 1998). Though the Fyn has been shown to play a role in astrocytes and microglia migration in response to a signal from neurons (Dey et al., 2008; Stuart et al., 2007), its role in microglial activation in response to seizures is unknown. Since we found a significant increase in the Fyn and pSrc-416 levels during early phase of epileptogenesis, and an increase in the numbers of reactive microglia in the hippocampus (Fig. 4), we speculated that Fyn also mediates neuroinflammatory response during epileptogenesis in addition to the loss of hilar neurons in the dentate gyrus. The microglia are resident immune cells of the brain which mediate innate immune response (Davis and Carson, 2012; Kettenmann et al., 2011). In the peripheral immune system, Fyn activates mast cells and lymphocytes and drives proinflammatory cytokines production (Tamura et al., 2001; Thomas and Brugge, 1997). The hippocampus and cortex contain a large amount of the SFK including Fyn (Salter and Kalia, 2004). Dysregulation of SFK in mast cells offers resistance to epileptogenesis in an epilepsy-resistant variant of mouse originating from epilepsy-prone EL mice colony (Kitaura et al., 2006). Therefore, we speculated that a similar mechanism may exist in kainate model of epileptogenesis. The Western blot and IHC analysis revealed a significant increase in nuclear Fyn and PKC δ levels in the hippocampus at 24h, and 7d post-SE in the *fyn*^{+/+} mice (Figs. 4, 5), which provide evidence for increased Src kinase activity during epileptogenesis. As expected, Fyn was completely absent in the *fyn*^{-/-} mice, however, we observed a marginal increase in pSrc-416 levels, which may suggest a partial role of other SFK (e.g. c-Abl tyrosine kinase) in epileptogenesis. The c-Abl has been implicated in reactive microgliosis and in neurodegeneration (Gonfloni et al., 2012; Maiani et al., 2011).

In concurrence with the Western blot results, we found a significant increase in Fyn and PKC δ staining in nuclei of microglia in the hippocampus during epileptogenesis in *fyn*^{+/+} mice (Fig. 4G–H, J; 5E–F). In a cell culture and animal model of PD, it has been shown that phosphorylated Fyn activates PKC δ in microglia, which in turn translocates to the nucleus

(Saminathan et al., 2011) and initiates transcription of proinflammatory cytokines either directly or in association with c-Abl or NFkB signaling components (Bujor et al., 2011; Gordon et al., 2016). It was shown that shRNA-mediated knockdown or genetic ablation of PKC δ in primary microglia abolished inflammogens-induced proinflammatory response in microglia, and also suppressed ROS and RNS production, and proinflammatory cytokines release (Gordon et al., 2016). Phosphorylated PKC δ also activates cytoplasmic subunit of the NOX2, which forms a functional complex with the membrane associated gp91^{phox} to activate NOX2 signaling pathway and drives ROS and RNS production (Bedard and Krause, 2007; Fontayne et al., 2002; Gordon et al., 2016). In our studies, since we observed PKC δ staining in the nuclei of reactive microglia in *fyn*^{+/+} mice and a reduction in seizure threshold as evident from EEG analysis, we speculated that proinflammatory cytokines and nitro-oxidative stress markers are also upregulated. As predicted, the proinflammatory mediators TNF- α , IL-1 β , IL-6, and IL-12 were upregulated in *fyn*^{+/+} mice. Likewise, the nitro-oxidative stress markers such as 4-HNE, gp91^{phox} and 3-NT levels were also upregulated. In the *fyn*^{-/-} mice, their levels were significantly reduced suggesting the proinflammatory role of Fyn in epileptogenesis. However, we found an increase in M1-like reactive microglia during post-SE in the *fyn*^{-/-} mice, in contrast to the control, suggesting a partial role of other signaling pathways. For example, toll-like receptor and HMGB1, I κ B kinase complex induced pathways involving Wnt signaling transduction pathway, cytokine signaling via JAK/STAT involving IFN- α and IFN- β in microglial activation (Gesuete et al., 2014; Kaminska et al., 2016; Walker et al., 2017).

It has also been suggested that Fyn KO may compensate the loss of SFK protein levels by upregulating the other members of SFK such as Yes and c-Abl (Grant et al., 1995; Lowell et al., 1994). We have not tested this concept in our study. However, the pre- and post- SE treatment regimen with saracatinib, in this study, was expected to target all members of the SFK to achieve robust anti-seizure and antiepileptogenic effects.

Increased levels of proinflammatory cytokines and nitro-oxidative stress molecules are known to cause neurodegeneration (Glass et al., 2010; Vezzani et al., 2011 and 2013), altered synaptic plasticity, and decreased seizure threshold in epilepsy models (Bozzi et al., 2011; Reddy and Kuruba, 2013). As described previously (Puttachary et al., 2016a), we used FJB and NeuN co-staining to confirm neurodegeneration in our model. There was a significant increase in neurodegeneration in the *fyn*^{+/+} mice when compared to the *fyn*^{-/-} mice (Fig. 9) suggesting Fyn's contribution to neurodegeneration during epileptogenesis. However, there was also increased neurodegeneration in the *fyn*^{-/-} mice, when compared to control, which may suggest the role of other SFK and/or a different mechanism. The SFKs are known to mediate NMDAR mediated hyperexcitability, which could have contributed to a moderate increase in neurodegeneration in *fyn*^{-/-} mice during post-SE. We did not test the compensatory mechanism in this study. However, we tested whether Fyn KO alone has any impact on hyperexcitability of neurons, measured by epileptiform spike rate and chronic seizures, a continuous video-EEG recording for 28d post-SE was undertaken. The results revealed a significant reduction in epileptiform spiking and spontaneous NCS in the *fyn*^{-/-}, in contrast to *fyn*^{+/+} mice, confirming the partial role of Fyn in epileptogenesis. Collectively, these results suggest that SFK, rather than Fyn alone, is a potential target to achieve anti-epileptogenic effect.

Previous studies have shown that SFK inhibitor, PP2 reduces frequency of epileptiform discharges in the hippocampal *in vitro* model (Salter and Kalia, 2004; Sanna et al., 2000). We used saracatinib in our *in vivo* studies. It is more potent than other SFK inhibitors, and has been in clinical trials for AD (Nygaard et al., 2015) and breast cancer (Gucalp et al., 2011). Saracatinib is an anilinoquinazoline compound which has high specificity for the tyrosine kinase domains of SFK (Hennequin et al., 2006). In these enzymes, an adenine moiety of ATP is bound to Src kinase domains through hydrogen bond networks. This allows chlorobenzodioxide moiety of quinazoline compounds to sit deep inside the hydrophobic pocket of SFK making number of hydrophobic contacts forming stronger inhibitor-enzyme complex. Moreover, the presence of C5 position on the quinazoline ring, which fits well into enzyme's ribose pocket, makes them even more selective for SFKs thereby increasing their binding affinity and potency (Ballard et al., 2005; Gibson et al., 2002; Hennequin et al., 2006). Furthermore, the SFKs has some enzyme residues present at the entrance of their pocket site, also known as gatekeepers, is another potential feature for the selectivity of quinazolines as inhibitors of SFKs. In contrast to SFKs, the ribose pocket in other kinases is open to wide range of compounds that makes them less selective in nature (Hennequin et al., 2006). In our experiments, saracatinib significantly reduced severity and duration of SE in the mouse kainate model suggesting its seizure modulatory effect. We further tested this in the rat kainate model of TLE and observed an anti-epileptogenic effect in four rats and a reduction in the numbers of spontaneous CS in others in contrast to the vehicle treated rats (Fig. 10), which all became epileptic and had >24 spontaneous CS in a month. These results imply that SFK play an important role in epileptogenesis and saracatinib could be a potential disease modifying agent for epilepsy.

In conclusion, the Fyn and PKC δ were upregulated in the microglia in hippocampus during epileptogenesis. Concurrently, proinflammatory cytokines and nitro-oxidative stressors were also upregulated to cause neurodegeneration, increased epileptiform spiking, and electrographic NCS. These changes were significantly reduced in the *fyn*^{-/-} mice suggesting the role of Fyn in epileptogenesis. The saracatinib pretreatment significantly reduced SE severity and the post-treatment modified epileptogenesis suggesting its therapeutic potential in seizure modulation and modification of epileptogenesis. Further experiments using microglial specific deletion of Fyn would help reveal the direct effect of microglia dependent neuroinflammatory processes in epileptogenesis.

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5. Highlights

- The Fyn-PKC δ pathway mediates neuroinflammation and epileptogenesis
- A Fyn/SFK inhibitor, saracatinib pretreatment significantly reduced SE severity
- Kainate-induced SE severity was significantly suppressed in *fyn* KO mice
- Neuroinflammatory and nitro-oxidative markers were reduced in *fyn* KO during post-SE
- Saracatinib post-treatment prevented or modified epileptogenesis in the rat model

Figure 1. The saracatinib (SAR) crossed the BBB, increased latency to seizure onset, and reduced seizure severity and mortality rate in C57BL/6J mice

The SAR (25 mg/kg) was administered orally 4h prior to a SHD of kainate (25 mg/kg, i.p.).

A, B) The comparison of the time course of behavioral SE between the groups (A) during the first 3h after kainate injection ($***p < 0.0001$, two way ANOVA between 1 and 1271 degrees of freedom, $F = 502.65$, $n = 15$ for each group). In B, each dot represents individual animal ($***p < 0.0001$, Mann-Whitney). C) The SAR treated group showed a significant reduction in the duration of CS ($***p < 0.0001$), and a very few mice reached stage 3 seizure after a prolonged period when compared with the vehicle treated group ($**p < 0.01$). D) The survival and mortality rate in SAR and the vehicle treated mice (Fisher's exact test).

E, F) The ion chromatogram showing the relative abundance of the SAR in the hippocampus. The LC-MS analysis confirmed that the SAR crossed the BBB and the SAR was persisted in the hippocampus at higher levels at 8h post-SE when compared to 28h post-administration ($***p < 0.001$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; Mann-Whitney test for figure B, C, and F.

Figure 2. Comparison of seizure severity and its duration, and mortality rate in *fyn*^{+/+} and *fyn*^{-/-} mice in SHD (A-C) and RLD (D-H) of kainate treatments

A) Each dot represents individual animal. The *fyn*^{-/-} mice showed a significant reduction in seizure severity compared to the *fyn*^{+/+} mice (*p<0.01, Mann-Whitney, n=10 for each group). B) The survival and mortality rate in *fyn*^{+/+} and *fyn*^{-/-} mice (*p<0.05, Fisher's exact test). Of the total 40% mortality, about 20% was observed after the diazepam treatment in the *fyn*^{-/-} mice. C) The latency to convulsive seizures (CS) onset, following a SHD of kainate, was significantly increased in *fyn*^{-/-} when compared to *fyn*^{+/+} mice (*p<0.05, Mann-Whitney). D) The comparison of the time course of behavioral SE between the groups during 2h established SE after mice reached first stage 3 seizure (***p<0.0001, two way ANOVA between 1 and 2072 degrees of freedom, F=839.67, n=36 for each group). In E, each dot represents individual animal (***p<0.0001, Mann-Whitney). F-H) The *fyn*^{-/-} mice showed a significant reduction in the duration of CS (F, ***p<0.001, Mann-Whitney), and there was no significant difference in the latency to the onset of CS. In *fyn*^{-/-} mice, 22% mortality was observed (8% during the SE and 14% post-diazepam), while in the *fyn*^{+/+} mice 10% of the mice died during the SE, but none died after the diazepam treatment (G, not significant, Fisher's exact test). H) There were no significant differences in the average numbers of RLD of kainate injections (5 mg/kg per injection at 30 min intervals) given to the *fyn*^{+/+} and *fyn*^{-/-} mice to achieve stage 3 seizure.

Figure 3. Real-time video-EEG recording and analyses of epileptiform spike and spontaneous NCS

A) A representative EEG trace recorded during the SE induced by a RLD of kainate is presented. The behavioral SE was terminated by diazepam (10 mg/kg, i.p.), 2h after the onset of first CS. B) A segment of the EEG trace covering the stages of seizures before and after the stage 5 seizure and the associated seizure stage-specific power bands are illustrated. The theta and delta power were increased in NCS, while the gamma power was increased during the stage 5 seizure, and the image represents the stage 5 behavior (jumping). C) The duration of CS for EEG seizures (stage 3) during the 2h established SE was significantly lower in the *fyn*^{-/-} mice when compared to the *fyn*^{+/+} mice (*p<0.05, Mann-Whitney test, n=6 for each group). D, E) Representative EEG traces and epileptiform spikes during the first 7d post-SE (D), and a typical spontaneous NCS episode with associated power bands (E). F, G) The comparison of NCS frequencies between the *fyn*^{-/-} and *fyn*^{+/+} mice during 28d post-SE (F, ***p<0.0001, two way ANOVA between 1 and 140 degrees of freedom, F= 35.17, n=6 per group; G, **p=0.0016, ***p<0.001, Mann-Whitney). H) An example of spike trains with spike clusters (<12s), a typical NCS episode (>12s), and isolated epileptiform spikes. I-J). The comparison of epileptiform frequencies between the *fyn*^{-/-} and *fyn*^{+/+} mice during 28d post-SE. (I, ***p<0.0001, two way ANOVA between 1 and 119 degrees of freedom, F= 70.61; J, **p=0.002, ***p=0.0006, Mann-Whitney test).

Figure 4. Fyn and pSrc-416 levels in the hippocampus during epileptogenesis

A–D) The Western blot analysis of Fyn and pSrc-416 at 4h, 24h, and 7d post-SE. The cytosolic and nuclear Fyn proteins were absent in the *fyn*^{-/-} mice, while they were significantly increased at 24h and 7d post-SE in *fyn*^{+/+} mice when compared to the naïve control. The pSrc-416 levels also increased significantly in the *fyn*^{+/+} mice at all time points, but no significant differences were observed in the pSrc-416 levels in the *fyn*^{-/-} compared to the control (D). However, when compared between the groups, it was reduced in the *fyn*^{-/-} mice at 24h and 7d post-SE (D) **p*<0.05, ***p*<0.01, ****p*<0.001; *n*=5–6. E–F): IHC images of the hilus of dentate gyrus demonstrating Fyn (yellow) co-localization in neurons in *fyn*^{+/+} mice. Fyn expression was significantly increased in the hilus of DG at all the time points when compared to the control. No significant difference was observed in CA1 and CA3 regions (***p*<0.01, ****p*<0.001). G–I): IHC images from CA3 region of the hippocampus showing Fyn immunoreactivity (green) at different time points (red, IBA1 for microglia; yellow/green, Fyn; blue, DAPI for nucleus). A representative high power view images (G- v, x, xi) of the microglia (red) that resembled M1/M2-like phenotype with or without Fyn (green/yellow) at 24h post-SE from the *fyn*^{+/+} and *fyn*^{-/-} are shown. The Images in the panel H represents Fyn immunoreactivity in the cytoplasm (i–iv) and its nuclear translocation (H, vi- green and viii-yellow) in the M1-like microglia (red). In the *fyn*^{+/+} mice, at all time points, the microglia had M1-like phenotype with a large cell body (hypertrophied) and thick cytoplasmic processes (e.g. H- iii, vii). In *fyn*^{-/-} mice, there were also M1 like microglia, but without Fyn (G, x). I) The M1-like microglia cell quantification. *Fyn*^{+/+} mice showed a significantly higher percentage of total M1-like phenotype, “reactive” microglia, at all time points when compared to their respective controls. However, a similar increase was observed in the percentage of M1-like microglia in *fyn*^{-/-} compared to the controls but this difference was not significant at 7d post-SE. J) Fyn was not detected in *fyn*^{-/-} mice. In *fyn*^{+/+} mice, there was a significant increase in both cytosolic and nuclear Fyn positive M1-like microglia at all the time points when compared to the control. One way ANOVA, **p*<0.05, ***p*<0.01, ****p*<0.001; *n*=5–6. Scale bar, all 100 μ m.

Figure 5. The PKC δ (cytosolic and nuclear), and pPKC δ -507 protein levels in the hippocampus during epileptogenesis

A–D): The Western blot analysis showed a significant increase in both the cytosolic and nuclear PKC δ levels, and also pPKC δ , in *fyn*^{+/+} at all the time points compared to the control. The cytosolic PKC δ levels were relatively decreased at 7d post-SE, but the nuclear PKC δ levels remained at the same levels at all the time points. In *fyn*^{-/-} mice, the cytosolic PKC δ levels were increased significantly at all time points, while pPKC δ -507 levels were increased significantly at 24h and 7d when compared to the control. However, their levels were significantly lower than the levels observed in *fyn*^{+/+} mice (*p<0.05, **p<0.01, ***p<0.001). Nuclear translocation of PKC δ in *fyn*^{-/-} was marginally increased, but this increase was much lower when compared to *fyn*^{+/+} mice. E–F): Immunohistochemistry images of CA1 region of the hippocampus showing PKC δ (green or yellow) immunoreactivity in the M1-like microglia (red). In the *fyn*^{+/+} mice, there were significant increase in the numbers of both cytoplasmic and nuclear PKC δ immunoreactive microglia at all time points when compared to the control. In *fyn*^{-/-} mice, there was a significant reduction in their numbers, at all time points, in both the nuclei and cytoplasm (and also the intensity of staining) of the M1-like microglia when compared to *fyn*^{+/+} mice (*p<0.01, **p<0.01, ***p<0.001). A high power view of the images show intense PKC δ staining in the nucleus and cytoplasm of M1-like microglia in *fyn*^{+/+} and *fyn*^{-/-} mice (E- v, x). One way ANOVA, *p<0.05, **p<0.01, ***p<0.001; n=5–6. Scale bar, 100 μ m.

Figure 6. The caspase-3 and cleaved caspase-3 levels in the hippocampus of *fyn*^{+/+} and *fyn*^{-/-} mice during epileptogenesis

In both groups, the caspase-3 levels were significantly increased at all time points. B) At 4h and 24h, the caspase-3 levels were significantly lower in *fyn*^{-/-} in contrast to the *fyn*^{+/+} mice. C) The cleaved caspase-3 levels also significantly increased at all time points in *fyn*^{+/+} when compared to the control, and when compared to the *fyn*^{-/-} mice, except at 7d post-SE. In *fyn*^{-/-} mice, cleaved caspase-3 levels also increased over time compared to the control, but their levels were significantly lower in *fyn*^{+/+} mice at 4h and 24h time points. One way ANOVA, *p<0.05, **p<0.01, ***p<0.001; n=5–6.

Figure 7. TNF- α , IL-1 β , and iNOS mRNA expression levels in the hippocampus, and serum IL-6 and IL-12 levels in *fyn*^{+/+} and *fyn*^{-/-} mice during epileptogenesis

A–C): In *fyn*^{+/+} mice, the TNF- α mRNA levels were significantly increased at all the time points, while the IL-1 β mRNA levels were increased at 24h and 7d post-SE when compared to the controls. In *fyn*^{-/-} mice, the TNF- α mRNA levels were significantly reduced at all time points, and IL-1 β levels at 24h post-SE when compared to the *fyn*^{+/+} mice. *p<0.05, **p<0.01, ***p<0.001; n=5–6. The iNOS mRNA levels were increased at all time points in *fyn*^{+/+} mice when compared to the control, especially at 4h post-SE, while its levels did not change significantly in *fyn*^{-/-} mice when compared to the control. However, there was a significant reduction in mRNA levels at 7d post-SE when compared between *fyn*^{+/+} and *fyn*^{-/-} mice. D–E): The serum IL-6 and IL-12 levels were significantly higher, except IL-12 at 7d post-SE, in *fyn*^{+/+} mice when compared to the controls at all time points. In *fyn*^{-/-} mice, the IL-6 levels were marginally increased at 24h, while the IL-12 levels were significantly higher at all time points when compared to the controls. When their levels were compared between the groups at various time points, there was a reduction in the IL-6 levels at 4h and 24h post-SE, but there were no significant differences in IL-12 levels between the groups. One way ANOVA, *p<0.05, **p<0.01, ***p<0.001. n=5–6.

Figure 8. The Western blot analysis of 4-HNE, gp91^{phox} and 3-NT in the hippocampus of *fyn*^{+/+} and *fyn*^{-/-} mice during epileptogenesis

A–D) Increased levels 4-HNE and gp91^{phox} were observed in the hippocampus at all the time points, in both groups (except at 7d for 4-HNE in *fyn*^{-/-}), when compared to their respective controls. The 3-NT levels were also increased at all the time points in both *fyn*^{-/-} and *fyn*^{+/+} mice when compared to their respective controls. At 24h and 7d post-SE, 4-HNE, gp91^{phox} and 3-NT levels were significantly reduced in *fyn*^{-/-} mice in contrast to *fyn*^{+/+} mice. *p<0.05, **p<0.01, ***p<0.001; n=5–6. E): Immunohistochemistry images of CA3 region of the hippocampus showing the 4-HNE and gp91^{phox} immunoreactive cells [IBA1

(red) and DAPI (blue) in all panels; 4-HNE (green/yellow) in panels i) to viii) and gp91^{phox} (green/yellow) in panels ix) to xvi) in E]. Scale bar, all 100 μ m. F–G): Cell quantification. The IBA1 positive microglial processes that were in close proximity to the pyramidal cell membranes in the CA3 region were counted. The 4-HNE and gp91^{phox} immunopositive cells were increased at all time points in *fyn*^{+/+} mice compared to the control. The maximum increase was observed at 24h post-SE. A significant reduction was observed in the gp91^{phox} positive microglia, but not the 4-HNE, at 7d post-SE in *fyn*^{-/-} when compared to *fyn*^{+/+} mice. In *fyn*^{-/-} mice, there was a significant increase in both 4-HNE and gp91^{phox} positive cells at all time point, except at 7d, compared to controls. One way ANOVA, *p<0.05, **p<0.01, ***p<0.001; n=5–6. Scale bar, all 100 μ m.

Figure 9. Neurodegeneration. FJB-NeuN co-staining in CA3 region of hippocampus
FJB positive cells (yellow) were observed in the neurons (red) of the pyramidal cell layer of CA3 in both *fyn*^{+/+} and *fyn*^{-/-} mice at all time points. The maximum neurodegeneration was observed in CA3 and hilus in both the groups at 24h post-SE (A, iii and vii, and B). A significant difference in the number of FJB positive cells was observed in the DG and in other areas of hippocampus at all time points in *fyn*^{+/+} and *fyn*^{-/-} when compared to their respective controls. High number of FJB positive cells were observed at 24h post-SE in *fyn*^{+/+} in CA3 (A, iii) but they were reduced at 7d post-SE. Similar results were observed in *fyn*^{-/-} where the neurodegeneration was diminished at 7d post-SE compared to other time points. In *fyn*^{-/-} mice, there was increased neurodegeneration in pyramidal layer of CA3 (A, viii) but this was significantly lower compared to *fyn*^{+/+} mice. Overall, neurodegeneration was reduced in *fyn*^{-/-} compared to *fyn*^{+/+} at all time points, especially at 24h and 7d post-SE, in all areas of hippocampus. One way ANOVA, **p*<0.05, ***p*<0.01, ****p*<0.001; *n*=5–6. Scale bar, all 100 μ m.

Figure 10. Saracatinib post-treatment in rats significantly reduced the number of spontaneous CS and the epileptiform spiking activity during 28 days post-SE period

A, B) The comparison between the numbers of spontaneous CS between the vehicle and SAR treated groups during the four weeks of study. There was a significant reduction in the number of seizure episodes in the SAR treated group when compared to the vehicle control (A, $***p<0.0001$, two way ANOVA between 1 and 280 degrees of freedom, $F=25.50$, $n=7$ for each group; B, $*p=0.017$, Mann-Whitney). C) Out of seven SAR treated rats, four did not develop epilepsy. Each dot represents the number of spontaneous CS episodes for the three rats that developed epilepsy. D, E) The comparison of epileptiform frequencies between the SAR and vehicle control rats during 28d post-SE. SAR treatment significantly reduced epileptiform spiking frequency compared to vehicle control (D, $***p<0.0001$, two way ANOVA between 1 and 307 degrees of freedom, $F=37.78$; E, $***p=0.0003$, Mann-Whitney test). F) There was no significant difference in the initial SE severity between the SAR and vehicle treated groups.