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BACKGROUND

Paediatric status epilepticus (SE) may result in long-term neurological outcomes. There is urgent need of animal models to study the pathologic mechanisms induced by unremitting seizures in the immature brain that lead to cognitive deficits and epilepsy. Since neuroinflammation and oxidative stress are ignited by activated glial cells and may contribute to disease development, we investigated this brain response in an infantile rat model of epileptic encephalopathy. This is a "condition in which epileptic activity itself may contribute to severe cognitive and behavioral impairments above and beyond what might be expected from the underlying pathology alone and it can worsen over time".¹

AIM

To characterize in-depth an infantile rat model of epileptic encephalopathy induced by *de novo* status epilepticus to study mechanisms and test novel disease-modifying treatments

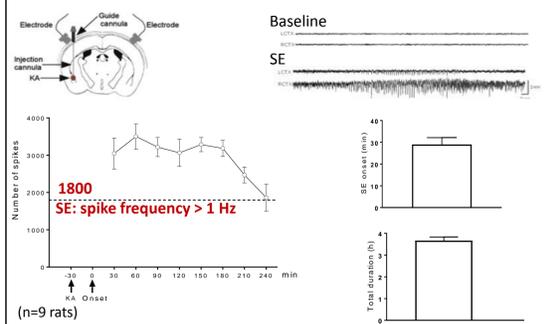
METHODS

EXPERIMENTAL MODEL: Status epilepticus (SE) was induced in postnatal day 13 (P13) male Sprague Dawley rats by unilateral injection of kainic acid (KA; 2µg/0.2µl) in the amygdala (from bregma, mm: anteroposterior, -1.4; lateral, -4.9).^{2,3} Controls were age-matched rats injected with PBS instead of KA. Four distinct groups of rats were prepared depending on the aim of the experiment, as follows: **Group 1-EEG analysis of SE:** P12 rat pups were stereotactically implanted under isoflurane anesthesia (1-3% isoflurane in a mixture of 70% N₂O-30% O₂) with an injection guide cannula positioned on top of *dura mater* for the intra-amygdala injection of KA, and bilateral cortical electrodes placed over the somatosensory cortex. The day after surgery, freely moving P13 rats were injected with KA after 30-min EEG baseline was established. SE was continuously video-EEG monitored for 4h. EEG analysis was done to assess SE severity and duration. **Group 2-Longitudinal study to monitor epilepsy and brain atrophy by MRI:** P13 rats were injected with KA and video-monitored for behavioral signs of SE (i.e. salivation, forelimb clonus, wild running and loss of posture) but not EEG recorded. SE-rats and their corresponding controls underwent T2-weighted MRI sequence to assess anatomic changes at PN22 and at 5 months post-SE (ex-vivo MRI). Rats were implanted with electrodes and video-EEG recorded for 2 weeks (24/7) at PN33 or PN65, and at 4.5 months post-SE to detect the frequency of spontaneous recurrent seizures. At the end of EEG recording, rats were killed and exposed to MRI. Brains were used for histological analysis. **Group 3-Longitudinal study to monitor cognitive deficits:** Rats were exposed to SE as described for Group 3, and then tested to Morris water maze task at PN25 and PN75. Rats were then implanted with electrodes and video-EEG recorder for 2 weeks (24/7) at 4.5 months post-SE. At the end of EEG recording, rats were killed and exposed to MRI. Brains were used for histological analysis. **Group 4-Histological and biochemical analysis of neuroinflammation and oxidative stress:** Rats were exposed to SE as described for Group 3, and then killed 6, 24, and 72h, or 7 days post-SE to perform immunohistochemistry or RT-qPCR of neuroinflammatory and oxidative stress markers. **MRI ANALYSIS:** RARE T2-weighted sequence of the whole brain (from the olfactory bulb to the cerebellum) was acquired using a 7T MRI. Rats were imaged under general anesthesia (1.4% isoflurane, in a mixture of 70%N₂O-30%O₂) and continuously monitored for changes in their respiratory rate. Body temperature was maintained at 36.5-37.5° C using a feed-back-regulated heating pad. The morphological images were obtained with a resolution of 130 x 130 x 130 µm³ (matrix = 231 x 96 x 138; FOV= 3 x 1.4 x 1.8 cm), repetition time of 2500 ms, effective echo time of 60 ms, and 1 average (total acquisition time: 34 min).

MORRIS WATER MAZE: A circular pool (diameter 150 cm, height 60 cm) was filled with water (25±1° C), and it was ideally divided in four quadrants. A squared platform was placed 1 cm below the water surface in the center of one of the quadrant. The platform was moved to a different quadrant during the second exposure to the behavioral test (PN75). The position of the platform was kept in the same quadrant throughout each behavioral test (PN25 and PN75). Each rat received one training session per day (4 swims) for 5 consecutive days. Each rat was allowed to swim until it found the platform or had swum unsuccessfully for 60 sec. In the latter case, the rat was guided to the platform by hand and its latency was recorded as 60 sec. After 10 sec rest on the platform, the rats were transferred to its home cage. The escape latency, i.e. the time to reach the hidden platform, was measured.⁴

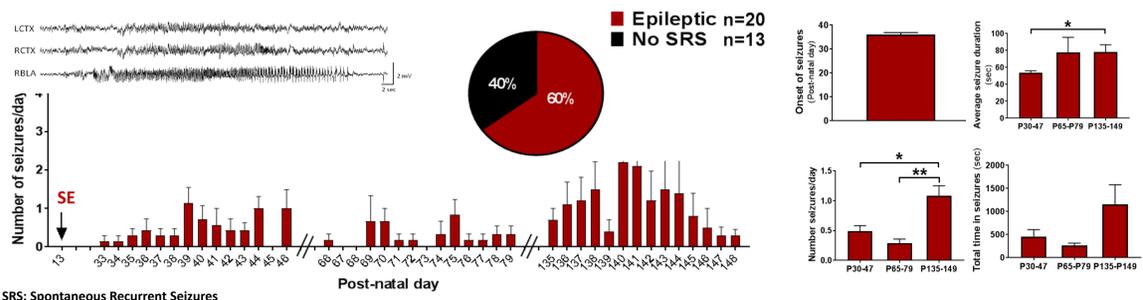
HISTOLOGICAL AND RT-qPCR ANALYSES. Immunohistochemistry and evaluation of neuronal cell loss: Rats were transcardially perfused 6, 24 and 72 h post-SE with PBS followed by 4% PAF in PBS. Brains were post-fixed in PAF, cryoprotected and then frozen at -50° C. Coronal sections of the brain (40 µm) were cut in a cryostat. We performed immunohistochemistry (IHC) using specific markers to detect astrocyte (GFAP) and microglia (CD-11b) activation, and Nrf2. Nissl staining and Fluoro-Jade were used to quantify neuronal cell loss.^{4,5} High-power non-overlapping fields (20X magnification) of the whole dorsal hippocampus were acquired, and the total number of Nissl-stained neurons was quantified. **RT-qPCR:** Rats were killed 24 h and 7 days post-SE, the hippocampus ipsilateral to the injected amygdala was dissected out on ice, frozen and used for measuring GFAP, CD-11b, HMGB1 and Nrf2 mRNAs.

EEG analysis of status epilepticus (SE)

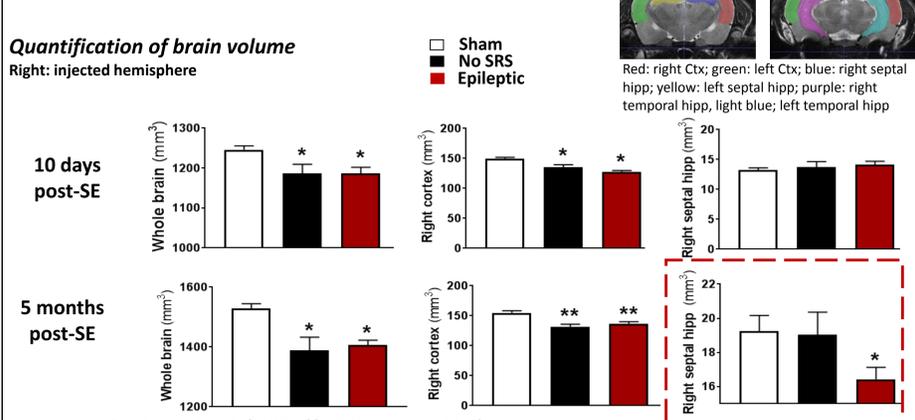


SE develops ~30 min after KA injection lasting for ~4h without inducing mortality.
During SE rat pups display motor seizures (masticatory movements and salivation, forelimb myoclonus, loss of posture, swimming motion).

Longitudinal EEG analysis of epilepsy development

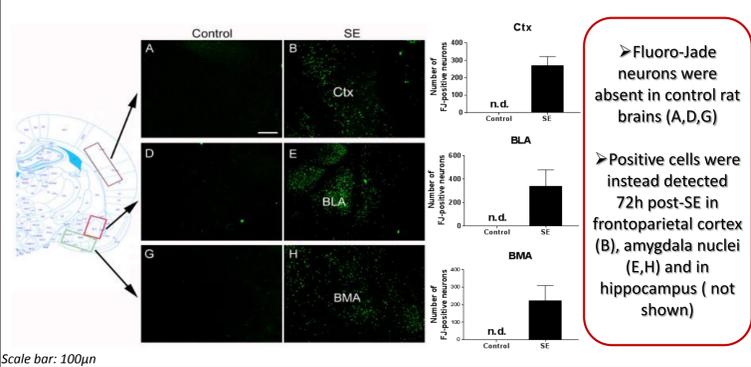


Longitudinal MRI analysis of brain atrophy

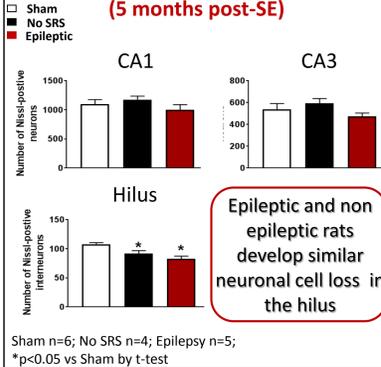


SE-exposed rats show whole brain and right cortex atrophy at 10 days post-SE, independently on whether they will develop spontaneous seizures. At 5 months post-SE, only epileptic rats show atrophy of the right septal hippocampus.
At 10 days post-SE, epileptic rats showed a significant reduction in the thickness of S1ULP as compared to rats without seizures and sham controls. S1ULP is a cortical region involved in the generation of spike-and-waves discharges.⁶ No changes in other cortical regions (cingulate, retrosplenial granular, perirhinal, primary somatosensory cortices) were observed.

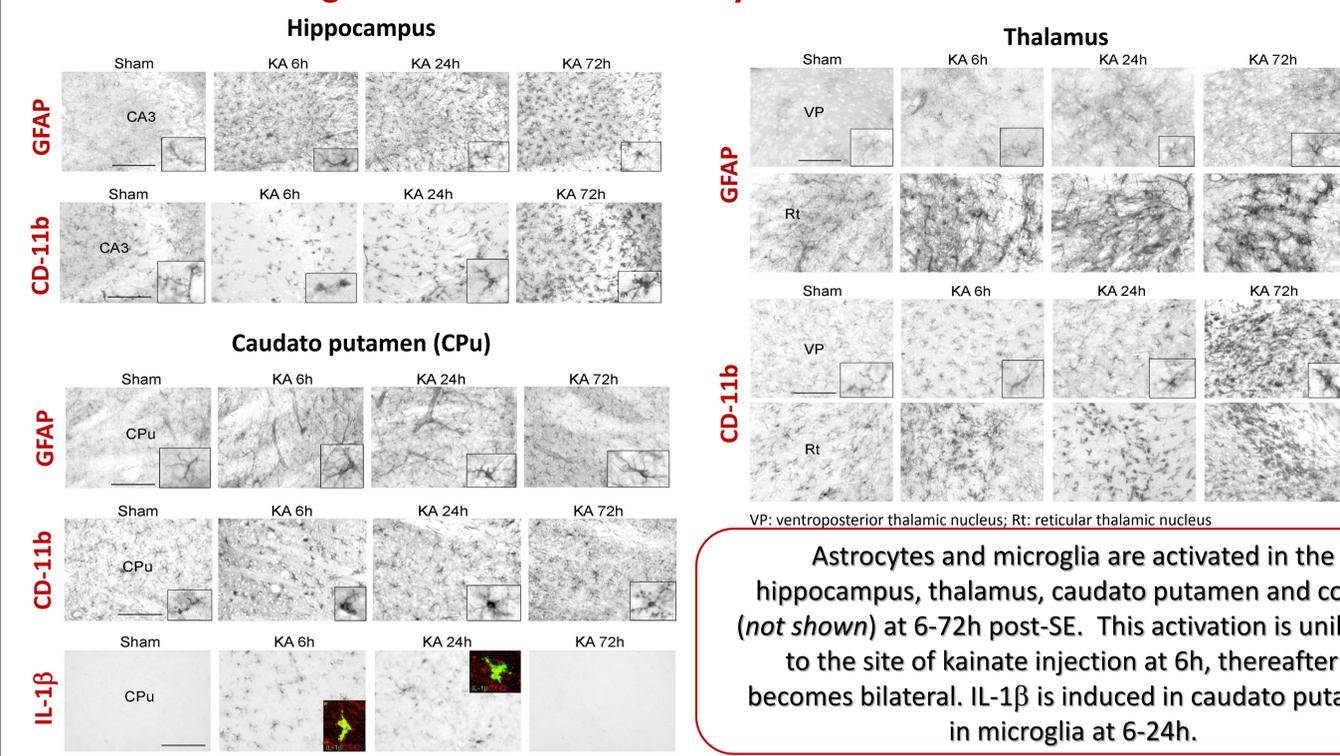
Neuronal cell loss following SE



Neuronal cell loss in the hippocampus (5 months post-SE)



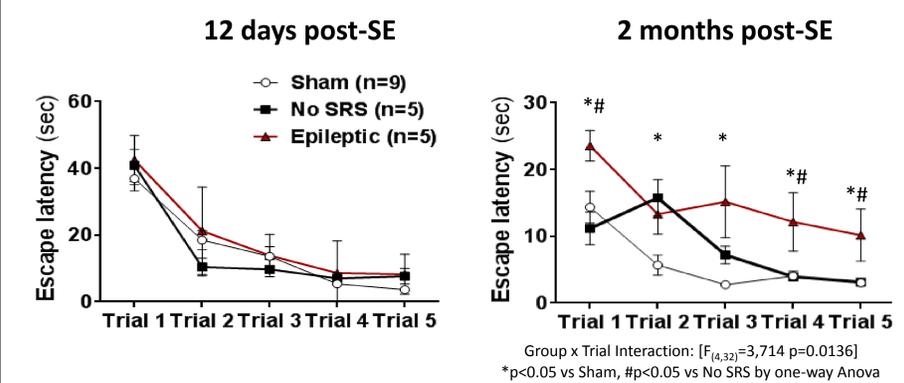
Histological and biochemical analysis of neuroinflammation and oxidative stress in the acute phase post-SE



RT-qPCR analysis showed an increase in mRNA levels of neuroinflammatory (GFAP, CD-11b, HMGB1) and oxidative stress (Nrf2) markers in the hippocampus 24h-1 week post-SE.

Astrocytes and microglia are activated in the hippocampus, thalamus, caudate putamen and cortex (not shown) at 6-72h post-SE. This activation is unilateral to the site of kainate injection at 6h, thereafter it becomes bilateral. IL-1β is induced in caudate putamen in microglia at 6-24h.

Longitudinal analysis of cognitive deficits: Morris Water Maze test



SE-exposed rats develop progressive memory deficits. 2 months post-SE, memory deficits are more pronounced in epileptic rats.

Summary

The rat model recapitulates some salient features of the clinical condition:
✓ age of animals (early childhood of human life)
✓ development of epilepsy
✓ cognitive decline and brain atrophy which worsen with epilepsy
Neuroinflammation and oxidative stress, two pathologic processes involved in the progression of epilepsy⁷⁻⁹, develop in the aftermath of SE.
MRI parameters prospectively differentiate rats with and without spontaneous seizures.

Conclusions

This model can be used for mechanistic studies and for testing novel drugs.
Since the model provides rats with and without spontaneous seizures (although all animals are similarly injured), it can be exploited to identify biomarkers of epileptogenesis.

Reference

- Berg, *Epilepsia*, 2011; 52 Suppl 1, 7-12
- Dunleavy et al, *Am J Pathol*, 2010; 176: 330-342
- Mesuret et al, *CNS Neurosci Ther*, 2014; 20:556-564
- Pascente et al, *Neurobiol Dis*, 2016; 93: 146-55
- Marcon et al, *Neurobiol Dis*, 2009; 34: 121-132
- Polack et al, *J Neurosci*, 2009; 29: 6590-6599
- Pauletti et al, *Brain*, 2017; 140: 1885-1899
- Iori et al, *Neurobiol Dis*, 2017; 99: 12-23
- Walker et al, *J Clin Invest*, 2017; 127: 2118-2132

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