Does angiogenesis play a role in the establishment of mesial temporal lobe epilepsy?

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Abstract

Mesial temporal lobe epilepsy (MTLE) is a focal epileptic disorder that is frequently associated with hippocampal sclerosis. This study investigated whether blocking angiogenesis prevents the development of seizures and hippocampal atrophy in the pilocarpine rat model of MTLE. To block angiogenesis, a subset of animals were given sunitinib orally. Continuous video recordings were performed to identify seizures. Brains were then extracted and sectioned, and hippocampal surfaces and angiogenesis were assessed. After a latent period of 6.6 ± 2.6 days, the sham-treated pilocarpine rats presented convulsive seizures, while the pilocarpine rats treated with sunitinib did not develop seizures. Sham-treated pilocarpine rats but not sunitinib-treated pilocarpine rats had significantly smaller hippocampi. Endothelial cell counts in sham-treated pilocarpine rats were significantly greater than in controls and sunitinib-treated pilocarpine rats. Blocking angiogenesis immediately following the initial insult in this animal model prevented thus angiogenesis and hippocampal atrophy and averted the development of clinical seizures.

Keywords

Angiogenesis; Epilepsy; Hippocampus; Pilocarpine rat model

1. Introduction

Epilepsy is one of the most common neurological disorders affecting up to 1% of the population worldwide (Moshé et al., 2015). It is estimated that approximately 50 per

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Conflict of interest

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100,000 children develop epilepsy each year (Neubauer et al., 2008). The symptomatic epilepsies represent a significant proportion of epileptic disorders and often develop as a consequence of an initial brain insult such as infection, febrile seizures, vascular accidents, or head trauma (Engel, 1996; Gloor, 1997; French et al., 1993; Salanova et al., 1994). The current pharmacological treatment of epilepsy is based on symptomatic management with anticonvulsant drugs that are ineffective in preventing the complex process of tissue remodeling known as epileptogenesis that follows the initial brain injury and finally leads to chronic epilepsy (Pitkanen and Sutula, 2002; Sutula, 2003). Dissecting the molecular and network mechanisms underlying epileptogenesis may guide the development of disease modifying agents and prevent this chronic condition that is often associated with significant morbidities.

Mesial temporal lobe epilepsy (MTLE) is a significant cause of childhood epilepsy. Among childhood epilepsy, MTLE is the most common symptomatic focal epileptic disorder with onset in late childhood to mid-adolescence. Up to 30% of children are medically intractable, often requiring surgical interventions (Engel, 1996; Wiebe et al., 2011). In MTLE, where seizures originate from within the hippocampus and parahippocampal structures such as the entorhinal cortex, and/or the temporal neocortex, there is an associated characteristic pattern of brain damage known as Ammon's horn sclerosis or mesial temporal sclerosis (Engel, 1996; Gloor, 1997; Wiebe et al., 2011).

Similar behavioral, electroencephalographic and histopathological findings can be reproduced in laboratory animals. Pharmacologically-induced status epilepticus (SE) with convulsant agents, such as pilocarpine, damages the brain and leads 1–4 weeks later to a chronic condition of recurrent limbic seizures that are poorly controlled by antiepileptic drugs (Chakir et al., 2006; Curia et al., 2008; Löscher and Köhling, 2010) and to hippocampal atrophy (Curia et al., 2008). Hence, in both humans and in animal models, MTLE is characterized by a seizure-free, latent period during which temporal lobe function becomes progressively altered until the appearance of the first seizure that marks the beginning of the epileptic condition.

Changes in temporal lobe excitability and connectivity, along with cells damages, are presumably triggered by the initial brain insult and represent major players in the epileptogenic processes occurring during the latent period (Pitkanen and Sutula, 2002; Sutula, 2003). However, brain injury also induces the activation of angiogenesis (Shibuya, 2009). Angiogenesis is presumed to have a role in the repair processes following brain injury (Xiong et al., 2010). Increasing evidence suggest that VEGF-mediated angiogenesis may create an adverse hyperexcitable milieu favoring epileptogenesis (Croll et al., 2004; Morin-Brureau et al., 2012; Marchi et al., 2012).

In this pilot study with a small number of animals, we used the pilocarpine rat model of MTLE to explore the effects of blocking angiogenesis on the development of chronic epilepsy and hippocampal atrophy. We hypothesized that blocking angiogenesis immediately following SE might decrease neovascularization within the seizure-onset zones and thus impact the development of chronic epilepsy and hippocampal atrophy in MTLE.

2. Methods

2.1. Pilocarpine rat model of mesial temporal lobe epilepsy

Male Sprague-Dawley rats (250–275 g; $n = 13$) were acquired from Charles River Laboratories (St-Constant, Qc, Canada) and were habituated to the environment for 72 h before pilocarpine treatment. On the day of injection, a subset ($n = 10$) were administered scopolamine methylnitrate (1 mg/kg, intraperitoneal; Sigma–Aldrich, Canada) and 30 min later a single dose of pilocarpine hydrochloride (380 mg/kg, intraperitoneal; Sigma–Aldrich, Canada) (Bortel et al., 2010; Lévesque et al., 2012). The behavior of the rats was scored according to the Racine scale (Racine, 1972); SE was defined as continuous stage 5 seizures. SE was terminated after 1 h by injection of diazepam (5 mg/kg, subcutaneous; CDMV, Canada) and ketamine (50 mg/kg, subcutaneous; CDMV, Canada) (Martin and Kapur, 2008). All experiments were conducted in accordance with the Canadian Council of Animal Care, and were approved by the local animal care committee.

2.2. Treatment blocking angiogenesis

Angiogenesis was blocked pharmacologically with sunitinib, a medication known to target the vascular endothelial growth factor (VEGF) pathway by inhibiting the phosphorylation of the VEGF receptors (VEGFR) (Rodriguez, 2007; Wood, 2012), and for which there is evidence for central nervous system penetration (Patyna and Peng, 2006; Speed et al., 2012; Medioni et al., 2007). Sunitinib maleate 20 mg/kg was given by oral gavage daily to a subset of pilocarpine rats ($n = 4$), starting within two hours after the status epilepticus was terminated and continuing for a total of 14 days. The sham-treated pilocarpine rats ($n = 6$) received a daily oral gavage of saline. Control rats with no pilocarpine or sunitinib treatment ($n = 3$) were also included. The dose of 20 mg/kg of sunitinib maleate used in this study was chosen to reflect a dose that has previously been demonstrated as safe, tolerable and effective (Patyna et al., 2008).

2.3. Evaluation of the latent period and seizure quantification in the chronic phase

After SE, rats were housed individually in custom-made plexiglas boxes (30 × 30 × 40 cm) and let habituate to the environment for 24 h. Continuous video monitoring (24 h per day) was performed from day 3 to day 21 after SE. For each animal, the latent period was defined as the number of days from the SE until the first spontaneous convulsive seizure was seen on video-monitoring (Biagini et al., 2006; Scorza et al., 2009).

2.4. Hippocampal surface measurements

On day 22 (i.e., 3 weeks after SE), the rats were deeply anesthetized with an intraperitoneal injection of 0.005 mg/kg xylazine, 0.05 mg/kg ketamine and 0.001 mg/kg acepromazine, and then transcardially perfused with 0.1 M phosphate buffered saline, followed by 4% paraformaldehyde. Brains were extracted and post-fixed in paraformaldehyde overnight at 4 °C, and then they were cryoprotected in 30% sucrose, and serially sectioned into 20 µm coronal sections.

Hematoxyline and eosin staining was performed on two different coronal brain sections of each animal at the level of the hippocampus. Each section was observed under microscope

(Leica DM4000B LED) at a magnification of 40×. For each section, overlapping microphotographs were taken using a digital camera attached to the microscope (Leica DFC450C). These were then stitched together using a panoramic image stitching software (Microsoft Research Image Composite Editor) to obtain a picture of the entire sections of the brain. Using an image analysis software (ImageJ) (Image Processing and Analysis in Java) (Schneider et al., 2012) converting the scale of the original pictures in mm², the surfaces of each hippocampus and each hemisphere were measured for each animal, and hippocampus to hemisphere ratio was calculated (Garcia-Finana et al., 2006). The mean of the right and the left measurements for each animal was used for the calculations.

2.5. Angiogenesis evaluation

To assess angiogenesis, immunohistochemistry was used to examine microvessel density. Sections were labeled with lectin (biotinylated isolectin B4) (Sigma–Aldrich: L2140, St-Louis, MO, USA) (dilution 20 µg/mL in 0.1 M tris-buffered saline [pH 7.4] and 0.5% Triton X-100, incubation time 2 h at room temperature) (Springer, 2010). To detect lectin binding, sections were incubated with streptavidin Alexa Fluor® 350 conjugate (Molecular Probes®, Life Technologies: S11249, Carlsbad, CA, USA) (dilution 1:300 in tris-buffered saline, incubation time 2 h at room temperature) (Ndode-Ekane et al., 2010). Then, these sections were rinsed with tris-buffered saline and cover slipped with Vectashield Mounting media containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories: H1200, Burlingame, CA, USA) that was used to visualize cell nuclei (Ndode-Ekane et al., 2010; Hallene et al., 2006). The endothelial cells count was assessed as per previously described methods (Hallene et al., 2006; Iwai et al., 2007; Noguchi et al., 2008; Rigau et al., 2007; Wintermark et al., 2013). Cells co-labeled with lectin and DAPI were identified as endothelial cells (Springer, 2010; Hallene et al., 2006). To estimate the density of microvessels, single immunoreactive endothelial cells were counted as individual microvessels; endothelial staining in large vessels with tunica media were disregarded in microvessel counts (Hallene et al., 2006; Iwai et al., 2007; Noguchi et al., 2008; Wintermark et al., 2013). For each animal, two fields of view in three different hippocampal regions of interest (i.e., the area 1 of cornu ammonis [CA1], the area 3 of cornu ammonis [CA3], and the dentate gyrus [DG]) were assessed on two different coronal brain sections at the level of the hippocampus. Using an image analysis software (ImageJ) (Image Processing and Analysis in Java) (Schneider et al., 2012) converting the scale of the original pictures in mm², the number of endothelial cells per mm² was measured for each animal. One investigator blinded to the groups took pictures of the respective fields of view at 100× magnification and assessed the endothelial cell count.

2.6. Statistical analysis

Considering the small animal samples, exploratory statistical analysis was performed to assess the differences in hippocampi surfaces, hippocampus to the whole hemisphere ratios, and endothelial cell count between the groups. Kruskal–Wallis tests followed by Dunnett's post-hoc multiple comparison tests with an alpha set up at 0.05 (95% confidence intervals) were performed to explore the differences between the control and experimental groups. A *p* value <0.05 was considered as statistically significant. All statistical analyses were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Evaluation of the latent period and seizure quantification in the chronic phase

The latent period, defined by the appearance of the first spontaneous convulsive seizure following SE, occurred after a mean of 6.6 ± 2.6 days in the sham-treated pilocarpine rats. All these animals developed seizures and they continued to have daily convulsive seizures (mean 7.9 ± 3.0 per day) throughout the monitoring period (Table 1). In contrast, none of the pilocarpine rats that were treated with sunitinib developed convulsive seizures during the three-week video-monitored period. None of the animals died during the experiments.

3.2. Hippocampi surface measurements

The sham-treated pilocarpine rats had hippocampi significantly smaller ($4.35 \pm 0.55 \text{ mm}^2$) ($p < 0.05$), compared to the controls ($4.96 \pm 0.32 \text{ mm}^2$) (Fig. 1). In the pilocarpine rats treated with sunitinib, the hippocampi surfaces improved ($4.58 \pm 0.55 \text{ mm}^2$), but they were not statistically different, compared to controls or sham-treated pilocarpine rats.

Hippocampus to hemisphere ratios followed the same trends as the hippocampal surfaces. The sham-treated pilocarpine rats had a smaller ratio (0.071 ± 0.008), compared to controls (0.078 ± 0.006) and the pilocarpine rats treated with sunitinib (0.074 ± 0.010), but it did not reach significance (Fig. 1).

3.3. Angiogenesis evaluation

For all three hippocampal regions of interest, the number of endothelial cells per mm^2 was significantly increased in the pilocarpine rats compared to the controls and the pilocarpine rats treated with sunitinib, but they were not different between the controls and the pilocarpine rats treated with sunitinib (Fig. 2). In the CA1 region of interest, the number of endothelial cells per mm^2 was significantly increased in pilocarpine rats (179.6 ± 48.9) ($p = 0.005$), compared to the controls (130.3 ± 35.4 , $p < 0.05$) and the pilocarpine rats treated with sunitinib (120.3 ± 38.9 , $p < 0.01$); these last two groups were not different. In the CA3 region of interest, the number of endothelial cells per mm^2 was significantly increased in the sham treated pilocarpine rats (210.1 ± 58.9), compared to controls (137.0 ± 49.6 , $p < 0.05$) and the pilocarpine rats treated with sunitinib (107.5 ± 74.4 , $p < 0.001$); these last two groups were not different. In the DG region of interest, the Kruskal–Wallis test came back significant ($p = 0.02$); the number of endothelial cells per mm^2 was increased in the sham-treated pilocarpine rats (185.9 ± 71.9), compared to controls (126.3 ± 46.2) and the pilocarpine rats treated with sunitinib (128.7 ± 39.4), but it did not reach statistical significance after Dunnett's post-hoc multiple comparison tests.

4. Discussion

A myriad of processes – including neuronal loss, axonal sprouting, alterations in receptor subunit composition, vascular changes, increased blood–brain barrier permeability, and inflammation – occur during the latent period in the rodent models of MTLE, such as the pilocarpine model; it is indeed assumed that these changes contribute to the establishment of the epileptogenic network that leads to and maintains the chronic epilepsy condition

(Pitkanen and Sutula, 2002; Sutula, 2003; Noebels et al., 2010). Although vascular modeling is known to occur in a number of neurologic conditions including stroke, traumatic brain injury and hypoxic-ischemic injury, its contribution to the pathogenesis of epilepsy is yet to be clarified (Krupinski et al., 1994; Morgan et al., 2007). Angiogenesis is presumed to have a role in the repair processes following brain injury (Xiong et al., 2010). However, increasing evidence suggest that VEGF-mediated angiogenesis may create an adverse hyperexcitable milieu favoring epileptogenesis (Croll et al., 2004; Morin-Brureau et al., 2012; Marchi et al., 2012). Using the pilocarpine rat model of MTLE, we confirmed in this pilot study with a small number of animals that rats exposed to SE not only develop spontaneous recurrent seizures and hippocampal atrophy compared to control rats, but they also have an increased vessel density within their hippocampi, which is indicative of activated angiogenesis. To the best of our knowledge, this is the first demonstration that directly blocking this activated angiogenesis with an anti-angiogenic agent (sunitinib) prevented the development of hippocampal atrophy and chronic seizures in the pilocarpine rats. These results hint at a pro-epileptogenic role for activated angiogenesis, and are in line with accumulating evidence suggesting that vascular remodeling occurs in MTLE and may be implicated in the establishment of epileptic networks (Morin-Brureau et al., 2012; Hallene et al., 2006; Rigau et al., 2007; Morgan et al., 2007; Marcon et al., 2009; Nicoletti et al., 2008). Indeed, not only have an increased vessel density, an upregulation of VEGF and its receptors, as well as an increased blood–brain barrier impairment been demonstrated within the hippocampus of tissue resected from epileptic patients with MTLE compared to tissue resected from non-epileptic patients (Rigau et al., 2007), but also the extent of vascular remodeling directly correlated with seizure frequency and not with other factors such as patient age, duration of epilepsy or extent of neuronal damage (Rigau et al., 2007).

In animal models of epilepsy, angiogenesis has been shown in a few reports to occur shortly after SE, and before the establishment of chronic epilepsy (Morin-Brureau et al., 2012; Hallene et al., 2006; Rigau et al., 2007; Hellsten et al., 2005; Marcon et al., 2009; Nicoletti et al., 2008). These studies have demonstrated that SE induces a pro-inflammatory state characterized by release of a myriad of cytokines that increases blood–brain barrier permeability and promotes neovascularization in both hippocampal and extrahippocampal structures (Marcon et al., 2009). Proliferation of endothelial cells, increased vessel density, increased expression of angiogenic factors such as vascular endothelial growth factor (VEGF) in neurons as well as VEGF tyrosine kinase receptors on blood vessels occur after SE and are most prominent within the CA3 region of the hippocampus (Morin-Brureau et al., 2012), as also demonstrated in our study with the most increased endothelial cells numbers in the CA3 region. The new vessels tend to be leaky resulting in blood-brain barrier impairment as demonstrated by loss of tight junctions that leads to extravasation of ions and serum proteins into the extracellular environment, triggers inflammation, leads to neuronal dysfunction and ultimately promotes seizures (Morin-Brureau et al., 2012; Hallene et al., 2006; Rigau et al., 2007).

Presently, medical management of seizures involves only a symptomatic approach that increases the brain's threshold for seizure generation but does not cure or alter the course of this morbid disease. Exploring the molecular mechanisms leading to the establishment of epileptogenic networks following a brain insult and further understanding the contribution of

activated angiogenesis may open the avenue for disease-modifying therapies that can prevent the establishment of chronic epilepsy. Based on our findings, blocking angiogenesis may prove to be one of these therapies to explore. Although anti-angiogenic agents have long been used for the treatment of cancers and more recently for macular degeneration and retinopathy of prematurity (Chang et al., 2012; Cammalleri et al., 2011), their role in the management of epilepsy has never been systematically explored *in vivo*.

Sunitinib is a VEGF tyrosine receptor antagonist that is currently being studied in patients with advanced renal cell carcinoma and works by inhibiting phosphorylation of the VEGF tyrosine receptor (Wood, 2012). It is an oral agent that is easy to administer and has good central nervous system bioavailability. In this pilot study, pilocarpine rats that were treated with sunitinib had lower endothelial cells count than the sham-treated pilocarpine rats, thus confirming that sunitinib blocked angiogenesis in these rats. Interestingly, the absence of angiogenesis in the sunitinib-treated rats translated into the absence of convulsive seizures and prevented the development of hippocampal atrophy, establishing an association between angiogenesis and the development of seizures and hippocampal atrophy. This would confirm some of the available *in vitro* studies suggesting this potential link. *In vitro*, blocking VEGF receptor signaling prevents epileptiform-induced vascularization and has been demonstrated to avoid the down-regulation of tight junctions that leads to seizure-induced increased blood-brain barrier dysfunction (Morin-Brureau et al., 2012). *In vitro* also, application of VEGF to rodent brain slices exposed to a potassium channel blocker (4-aminopyridine) in the absence of magnesium reduces the duration of both interictal and ictal activity (Cammalleri et al., 2011). This “antiepileptic” effect of VEGF appears to be even more pronounced in chronically epileptic tissue (McCloskey et al., 2005). *In vivo*, exogenous application of VEGF has been shown to protect against SE-induced neuronal loss within vulnerable regions such as the hippocampus (Nicoletti et al., 2008). A long-term follow-up to our study is warranted to confirm that the effect of blocking angiogenesis on chronic epilepsy and hippocampi persist at long-term and to rule-out the possibility of a slower degeneration of the hippocampi that is not yet evidenced 3 weeks after SE, as it is usually described in this rat model (Liu et al., 1994).

These results, albeit very preliminary, are interesting as they demonstrate the potential of using anti-VEGF treatment as a disease-modifying approach in epilepsy. It does not provide a definitive answer to our hypothesis as it is performed only in a small number of animals and longer-term follow-up would be needed. However, it does suggest, for the first time, that blocking angiogenesis may represent an innovative therapeutic avenue for prevention of chronic epilepsy. Although angiogenesis has been confirmed in a number of previous studies to play an important role in epileptogenesis (Morin-Brureau et al., 2012; Nnode-Ekane et al., 2010; Noguchi et al., 2008; Morgan et al., 2007; Hellsten et al., 2005; Marcon et al., 2009), no studies have explored, to our knowledge, the possible therapeutic effect of blocking its occurrence. Studying the impact of blocking angiogenesis on neurogenesis, neuroinflammation, astrogliosis, dispersion of the granular cell layer, sprouting, blood–brain barrier permeability, and VEGF expression, is now of utmost importance to further explore the link between angiogenesis and epileptogenesis. In addition, investigating the same hypothesis in other animal models of epilepsy (Löscher, 2011) may also be of interest.

Despite the novelty of these preliminary findings, there are a number of limitations of this study that need to be highlighted in addition to the small sample size. In this study, we attributed the anti-epileptogenic mechanism of sunitinib to its role in preventing angiogenesis. However, we did not address the possibility that sunitinib might have resulted in the absence of seizures by modifying or reducing the impact of the initial insult (status epilepticus) via its anti-VEGF property or other mechanisms. Although there is some evidence that sunitinib may have a partial neuroprotective mechanism through inhibition of nitric oxide production (Cui et al., 2014), there is currently no evidence from the literature, to our knowledge, that sunitinib has any anti-convulsant role that may have allowed to modulate the initial status epilepticus. However, only studies with EEG recordings performed during and after the status epilepticus could exclude this possibility with certainty.

Another important limitation of our study was that since the rats were only video-monitored, the presence of non-convulsive seizures cannot be ruled out and this would have to be explored further with studies using depth-electrode and continuous video-EEG monitoring. Thus, although this pilot study demonstrated that rats treated with sunitinib following SE did not develop convulsive seizures, we cannot claim with certainty without EEG recording that these rats were not “epileptic” per se. However, the absence of hippocampal atrophy in this treatment group rendered it less probable that these rats had purely non-convulsive seizures.

Finally, it may be argued that the use of lectin in the quantification of angiogenesis in this study may be inaccurate due to the fact that lectin labels both blood vessels as well as microglia. However, it is well established that microglia have a distinct morphology from vessels, which permits their exclusion in the endothelial cell count (Shaikh et al., 2015; Wintermark et al., 2015).

5. Conclusion

In conclusion, this study suggests that angiogenesis may play a vital pro-epileptogenic role following brain injury and blocking it may avert the development of the chronic epileptic condition. These results open the avenue for exploring further the role of innovative disease-modifying therapies targeting angiogenesis that can be instituted shortly after brain injury to prevent the development of epilepsy, rather than only relying on symptomatic medical antiepileptic management. Determining if blocking angiogenesis may represent an innovative therapeutic approach in children and adults with refractory seizures is a worthy challenge, but extreme caution should be used in the choice of the anti-angiogenic agent before its use in infants and children so as to ensure that it does not have any deleterious effects on the normal developing brain.

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Abbreviations

CA3	cornu ammonis 3
DAPI	4,6-diamidino-2-phenylindole
DG	dentate gyrus
MTLE	mesial temporal lobe epilepsy
SE	status epilepticus
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

References

- Biagini G, Baldelli E, Longo D, Pradelli L, Zini I, Rogawski MA, Avoli M. Endogenous neurosteroids modulate epileptogenesis in a model of temporal lobe epilepsy. *Exp Neurol*. 2006; 201:519–524. [PubMed: 16780839]
- Bortel A, Lévesque M, Biagini G, Gotman J, Avoli M. Convulsive status epilepticus duration as determinant for epileptogenesis and interictal discharge generation in the rat limbic system. *Neurobiol Dis*. 2010; 40:478–489. [PubMed: 20682341]
- Cammalleri M, Martini D, Ristori C, Timperio AM, Bagnoli P. Vascular endothelial growth factor up-regulation in the mouse hippocampus and its role in the control of epileptiform activity. *Eur J Neurosci*. 2011; 33:482–498. [PubMed: 21198982]
- Chakir A, Fabene PF, Ouazzani R, Bentivoglio M. Drug resistance and hippocampal damage after delayed treatment of pilocarpine-induced epilepsy in the rat. *Brain Res Bull*. 2006; 71:127–138. [PubMed: 17113938]
- Chang JH, Garg NK, Lunde E, Han KY, Jain A, Azar DT. Corneal neovascularization: an anti-VEGF therapy review. *Surv Ophthalmol*. 2012; 57:415–429. [PubMed: 22898649]
- Croll SD, Goodman JH, Scharfman HE. Vascular endothelial growth factor (VEGF) in seizures: a double-edged sword. *Adv Exp Med Biol*. 2004; 548:57–68. [PubMed: 15250585]
- Cui W, Zhang ZJ, Hu SQ, Mak SH, Xu DP, Choi CL, Wang YQ, Tsim WK, Lee MY, Rong JH, Han YF. Sunitinib produces neuroprotective effect via inhibiting nitric oxide overproduction. *CNS Neurosci Ther*. 2014; 20:244–252. [PubMed: 24393200]
- Curia G, Longo D, Biagini G, Jones RS, Avoli M. The pilocarpine model of temporal lobe epilepsy. *J Neurosci Methods*. 2008; 172:143–157. [PubMed: 18550176]
- Engel J Jr. Introduction to temporal lobe epilepsy. *Epilepsy Res*. 1996; 26:141–150. [PubMed: 8985696]
- French JA, Williamson PD, Thadani VM, Darcey TM, Mattson RH, Spencer SS, Spencer DD. Characteristics of medial temporal lobe epilepsy: I. Results of history and physical examination. *Ann Neurol*. 1993; 34:774–780. [PubMed: 8250525]
- Garcia-Finana M, Denby CE, Keller SS. Degree of hippocampal atrophy is related to side of seizure onset in temporal lobe epilepsy. *AJNR Am J Neuroradiol*. 2006; 27:1046–1052. [PubMed: 16687541]
- Gloor, P. *The Temporal Lobe and Limbic System*. Oxford University Press; New York: 1997.
- Hallene KL, Oby E, Lee BJ, Santaguida S, Bassanini S, Cipolla M, Marchi N, Hossain M, Battaglia G, Janigro D. Prenatal exposure to thalidomide, altered vasculogenesis, and CNS malformations. *Neuroscience*. 2006; 142:267–283. [PubMed: 16859833]
- Hellsten J, West MJ, Arvidsson A, Ekstrand J, Jansson L, Wennström M, Tingstrom A. Electroconvulsive seizures induce angiogenesis in adult rat hippocampus. *Biol Psychiatry*. 2005; 58:871–878. [PubMed: 16043138]

- Iwai M, Cao G, Yin W, Stetler RA, Liu J, Chen J. Erythropoietin promotes neuronal replacement through revascularization and neurogenesis after neonatal hypoxia/ischemia in rats. *Stroke*. 2007; 38:2795–2803. [PubMed: 17702962]
- Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke*. 1994; 25:1794–1798. [PubMed: 7521076]
- Lévesque M, Salami P, Gotman J, Avoli M. Two seizure-onset types reveal specific patterns of high-frequency oscillations in a model of temporal lobe epilepsy. *J Neurosci Off J Soc Neurosci*. 2012; 32:13264–13272.
- Löscher W, Köhling R. Functional, metabolic, and synaptic changes after seizures as potential targets for antiepileptic therapy. *Epilepsy Behav*. 2010; 19:105–113. [PubMed: 20705520]
- Löscher W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure*. 2011; 20:359–368. [PubMed: 21292505]
- Liu Z, Nagao T, Desjardins GC, Gloor P, Avoli M. Quantitative evaluation of neuronal loss in the dorsal hippocampus in rats with long-term pilocarpine seizures. *Epilepsy Res*. 1994; 17:237–247. [PubMed: 8013446]
- Marchi N, Granata T, Ghosh C, Janigro D. Blood–brain barrier dysfunction and epilepsy: pathophysiologic role and therapeutic approaches. *Epilepsia*. 2012; 53:1877–1886. [PubMed: 22905812]
- Marcon J, Gagliardi B, Balosso S, Maroso M, Noé F, Morin M, Lerner-Natoli M, Vezzani A, Ravizza T. Age-dependent vascular changes induced by status epilepticus in rat forebrain: implications for epileptogenesis. *Neurobiol Dis*. 2009; 34:121–132. [PubMed: 19320047]
- Martin BS, Kapur J. A combination of ketamine and diazepam synergistically controls refractory status epilepticus induced by cholinergic stimulation. *Epilepsia*. 2008; 49:248–255. [PubMed: 17941842]
- McCloskey DP, Croll SD, Scharfman HE. Depression of synaptic transmission by vascular endothelial growth factor in adult rat hippocampus and evidence for increased efficacy after chronic seizures. *J Neurosci*. 2005; 25:8889–8897. [PubMed: 16192378]
- Medioni J, Cojocarasu O, Belcaceres JL, Halimi P, Oudard S. Complete cerebral response with sunitinib for metastatic renal cell carcinoma. *Ann Oncol*. 2007; 18(7):1282–1283. [PubMed: 17675397]
- Morgan R, Kreipke CW, Roberts G, Bagchi M, Rafols JA. Neovascularization following traumatic brain injury: possible evidence for both angiogenesis and vasculogenesis. *Neurol Res*. 2007; 29:375–381. [PubMed: 17626733]
- Morin-Brureau M, Rigau V, Lerner-Natoli M. Why and how to target angiogenesis in focal epilepsies. *Epilepsia*. 2012; 53:64–68. [PubMed: 23134498]
- Moshé SL, Perucca E, Ryvlin P, Tomson T. Epilepsy: new advances. *Lancet*. 2015; 385:884–898. [PubMed: 25260236]
- Ndode-Ekane XE, Hayward N, Gröhn O, Pitkänen A. Vascular changes in epilepsy. *Neuroscience*. 2010; 166:312–332. [PubMed: 20004712]
- Neubauer BA, Gross S, Hahn A. Epilepsy in childhood and adolescence. *Dtsch Arztebl Int*. 2008; 105(17):319–327. [PubMed: 19629244]
- Nicoletti JN, Shah SK, McCloskey DP, Goodman JH, Elkady A, Atassi H, Hylton D, Rudge JS, Scharfman HE, Croll SD. Vascular endothelial growth factor is up-regulated after status epilepticus and protects against seizure-induced neuronal loss in hippocampus. *Neuroscience*. 2008; 151:232–241. [PubMed: 18065154]
- Noebels JL, Avoli M, Rogawski M, Olsen R, Delgado-Escueta AV. Jasper's basic mechanisms of the epilepsies workshop. *Epilepsia*. 2010; 51:1–5.
- Noguchi T, Yoshiura T, Hiwatashi A, Togao O, Yamashita K, Nagao E, Shono T, Mizoguchi M, Nagata S, Sasaki T, Suzuki SO, Iwaki T, Kobayashi K, Mihara F, Honda H. Perfusion imaging of brain tumors using arterial spin-labeling: correlation with histopathologic vascular density. *AJNR Am J Neuroradiol*. 2008; 29:688–693. [PubMed: 18184842]
- Patyna S, Peng G. Distribution of sunitinib and its active metabolite in brain and spinal cord tissue following oral or intravenous administration in rodents and monkeys (Abstract 56). *Eur J Cancer*. 2006; 4(Suppl):21.

- Patyna S, Arrigoni C, Terron A, Kim TW, Heward JK, Vonderfecht SL, Denlinger R, Turnquist SE, Evering W. Nonclinical safety evaluation of sunitinib: a potent inhibitor of VEGF, PDGF, KIT, FLT3, and RET receptors. *Toxicol Pathol.* 2008; 36(7):905–916. [PubMed: 18981453]
- Pitkanen A, Sutula TP. Is epilepsy a progressive disorder? Prospects for new therapeutic approaches in temporal-lobe epilepsy. *Lancet Neurol.* 2002; 1:173–181. [PubMed: 12849486]
- Racine RJ. Modification of seizure activity by electrical stimulation. II Motor seizure. *Electroencephalogr Clin Neurophysiol.* 1972; 32:281–294. [PubMed: 4110397]
- Rigau V, Morin M, Rousset MC, de Bock F, Lebrun A, Coubes P, Picot MC, Baldy-Moulinier M, Bockaert J, Crespel A, Lerner-Natoli M. Angiogenesis is associated with blood–brain barrier permeability in temporal lobe epilepsy. *Brain.* 2007; 130:1942–1956. [PubMed: 17533168]
- Rodriguez E. Biology and clinical applications of angiogenesis inhibition in malignant disease. *Mol Oncol Rep.* 2007; 1:2–3.
- Salanova V, Markand ON, Worth R. Clinical characteristics and predictive factors in 98 patients with complex partial seizures treated with temporal resection. *Arch Neurol.* 1994; 51:1008–1013. [PubMed: 7944998]
- Schneider CA, Rasband WS, Eliceiri KW. NIH image to imageJ: 25 years of image analysis. *Nat Methods.* 2012; 9:671–675. [PubMed: 22930834]
- Scorza FA, Arida RM, da Naffah-Mazzacoratti MG, Scerni DA, Calderazzo L, Cavalheiro EA. The pilocarpine model of epilepsy: what have we learned? *An Acad Bras Cienc.* 2009; 81:345–365. [PubMed: 19722008]
- Shaikh H, Lechpammer M, Jensen FE, Warfield SK, Hansen AH, Kosaras B, Shevell M, Wintermark P. Increased brain perfusion persists over the first month of life in term asphyxiated newborns treated with hypothermia: does it reflect activated angiogenesis? *Transl Stroke Res.* 2015; 6:224–233. [PubMed: 25620793]
- Shibuya M. Brain angiogenesis in developmental and pathological processes. *FEBS J.* 2009; 276:4638–4639.
- Speed B, Bu HZ, Pool WF, Peng GW, Wu EY, Patyna S, Bello C, Kang P. Pharmacokinetics, distribution, and metabolism of [¹⁴C] sunitinib in rats, monkeys, and humans. *Drug Metab Dispos.* 2012; 40(3):539–555. [PubMed: 22180047]
- Springer ML. Assessment of myocardial angiogenesis and vascularity in small animal models. *Methods Mol Biol.* 2010; 660:149–167. [PubMed: 20680818]
- Sutula TP. Mechanisms of epilepsy progression: current theories and perspectives from neuroplasticity in adulthood and development. *Epilepsy Res.* 2003; 60:161–171.
- Wiebe S, Blume WT, Girvin JP, Eliasziw M. Effectiveness and efficiency of surgery for temporal lobe epilepsy study group. A randomized, controlled trial of surgery for temporal-lobe epilepsy. *N Engl J Med.* 2011; 345:311–318.
- Wintermark P, Lechpammer M, Warfield SK, Kosaras B, Takeoka M, Poduri A, Madsen JR, Bergin AM, Whalen S, Jensen FE. Perfusion imaging of focal cortical dysplasia using arterial spin labeling: correlation with histopathological vascular density. *J Child Neurol.* 2013; 28:1474–1482. [PubMed: 23696629]
- Wintermark P, Lechpammer M, Kosaras B, Jensen FE, Warfield SK. Brain perfusion is increased at term in the white matter of very preterm newborns and newborns with congenital heart disease: may this reflect activated angiogenesis? *Neuropediatrics.* 2015; 46:344–351. [PubMed: 26340765]
- Wood L. Sunitinib malate for the treatment of renal cell carcinoma. *Expert Opin Pharmacother.* 2012; 13:1323–1336. [PubMed: 22607009]
- Xiong Y, Mahmood A, Chopp M. Angiogenesis neurogenesis and brain recovery of function following injury. *Curr Opin Investig Drugs.* 2010; 11:298–308.

Fig. 1.

Hippocampal surface. (A–C) Representative hematoxylin and eosin-stained coronal brain section at the level of the hippocampus, magnification 40 $\times$, of (A) control rats with no pilocarpine or sunitinib treatment, (B) sham-treated pilocarpine rats, and (C) pilocarpine rats treated with sunitinib, (D) Hippocampal surface, in mm² and (E) hippocampus to hemisphere ratio. SHAM VEH, control rats with no pilocarpine or sunitinib treatment; PILO VEH, sham-treated pilocarpine rats, i.e., treated with saline; PILO SUN, pilocarpine rats treated with sunitinib. Mean $\pm$ SEM. Significance: * $p < 0.05$. The hippocampal surface was significantly reduced in the sham-treated pilocarpine rats, compared to the controls; the surface improved in the pilocarpine rats treated with sunitinib. The hippocampus to hemisphere ratio followed the same trend.

Fig. 2.

Angiogenesis. (A–C) Representative lectin-stained area of regions of interest in the cornu ammonis 3 (CA3) region of interest, magnification 100×, of (A) control rats with no pilocarpine or sunitinib treatment, (B) sham-treated pilocarpine rats, and (C) pilocarpine rats treated with sunitinib, (D–F) Endothelial cells count per mm², in (D) cornu ammonis [CA1], (E) cornu ammonis [CA3], and (F) dentate gyrus [DG]. SHAM VEH, control rats with no pilocarpine or sunitinib treatment; PILO VEH, sham-treated pilocarpine rats, i.e., treated with saline; PILO SUN, pilocarpine rats treated with sunitinib. Mean ± SEM. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The endothelial cells count was increased in the sham-treated pilocarpine rats, compared to controls and the pilocarpine rats treated with sunitinib; these last two groups were not different.

Table 1

Evaluation of the latency period and seizure quantification in the chronic phase in the pilocarpine rats. Continuous video monitoring (24 h per day) was performed from day 3 to day 21 after status epilepticus (SE). The latent period was defined as the number of days from the SE until the first spontaneous convulsive seizure was seen on video-monitoring.

	Sham-treated pilocarpine rats (<i>n</i> = 6)	Sunitinib-treated pilocarpine rats (<i>n</i> = 4)
Duration of the latent period, days	6.6 ± 2.6	N/A
Number of convulsive seizures per day, mean	7.9 ± 3.0	0