Peer Review File

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The paper focuses on the role of microtubules on the seizures induced in the lithium-pilocarpine model in rats. Molecular essays have been conducted to evaluate a and b tubulin role in epileptic seizures. Treatment with microtubule-active agents were performed i.c.v. to explore the eventual protective effects on status epilepticus.

Major Points

• In the introduction, it is crucial to understand if and why microtubules have been implicated in epileptic phenomena. Only two studies were reported to support this thesis, but literature has explored this topic so far and this deserves to be addressed. The authors indeed state that actine cytoskeleton is implicated in SE and also a downregulation of a and b tubulin was found. Actually, studies have specifically correlated levels of major components of both actin and mictrotubule cytoskeleton to network remodelling in hippocampal epilepsy, such as MAP 2, but also the up-regulation of genes associated with tubulin in human epileptic tissues. See: [(Tang L, Lu Y, Zheng W, Li Y (2014) ; Sato K, Abe K (2001) ; Machado RA, Benjumea-Cuartas V, Zapata Berruecos JF, et al (2019) ; Xi ZQ, Wang XF, Shu XF, et al (2011)]. In addition, pharmacological manipulation of cytoskeleton has been performed providing interesting effects such as anti-seizure effects of pharmacological manipulation of Tau hyper-phosphorilation in several chronic models of epilepsy [(Liu S, Shen Y, Shultz SR, et al (2017); Jones NC, Nguyen T, Corcoran NM, et al (2012); Liu SJ, Zheng P, Wright DK, et al (2016)] or the application of nocodazole, a microtubule stabilyzing agent in in vivo and in vitro electrophysiological models of epilepsy (Carletti et al. 2016)

Reply: Thanks for professor Reviewer A's excellent points. we have modified our text as advised (see Page 6 line 104-107; Page 7 line 121-125)".

• In the methodological procedures, authors state that the pilocarpine-induced SE is achieved after 25 mg/Kg of pilocarpine is administered, but not in all animals. Therefore, when rats do not reach score 4-5 of Racine scale, a further 10mg/Kg every 30 min is administered until SE or a maximal 60/Kg dose is reached. Though, as in Curia et al 2010 review, the studies using lithium-pilocarpine model show a 100% effectiveness of epileptogeninc induction of SE in Rats and the maximal dose of pilocarpine is 30 mg/kg. What is the percentage of rats not reaching the SE in the authors results? What about mortality rate of the rats receiving 60 mg/Kg dose, considering that at 30 mg/kg it was already reported a mortality over 90% (Curia et al. 2010)? Authors in this paper report only a general mortality of 20.6%, without discriminating on the dose actually administered.

Reply: Thanks for raising this good question. Actually, we want to apologize first that there is a clerical error in the methodological procedure, which we use 20 mg/Kg of pilocarpine for the first dose instead of 25 mg/Kg of pilocarpine (already corrected in

the text, see page 8, line 156). To our knowledge, a single high dose (320-400 mg/kg) of pilocarpine didn't not always induce SE, and the mortality rate was high (1). Clifford et al found that rats pretreated with lithium chloride needed a much lower dose of pilocarpine (30 mg/kg) to induce SE (2), but the mortality rate was still up to 45%. Glien et al administrated a repeated low-dose (10mg/Kg) of pilocarpine, the mortality rate significantly decreased to below 10% (3). In our lab's preliminary experiments, we referred to Glien's method and found that although the mortality rate was low, the successful modeling rate was still only 61%, which was not satisfying enough. Thus, we made some modifications, changing the first dose to 20 mg/Kg, followed by repeated low-dose (10mg/Kg) administration, and we found that this method of triggering SE exhibited higher successful rate (up to 90%) and lower mortality (21.2%), which we thought was better than the traditional way. Based on this method, our lab has already published a few papers (4, 5). We also added some data in the animal modeling result part (see page 14, line 300-302) as advised, to make our experiments and results clearer and more precise. Thanks again for raising this issue.

1. Turski L, Ikonomidou C, Turski WA, et al. Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. Synapse. 1989;3(2):154-71.
2. Clifford DB, Olney JW, Maniotis A, et al. The functional anatomy and pathology of lithium-pilocarpine and high-dose pilocarpine seizures. Neuroscience. 1987;23(3):953-68.
3. Glien M, Brandt C, Potschka H, et al. Repeated low-dose treatment of rats with pilocarpine: low mortality but high proportion of rats developing epilepsy. Epilepsy Res. 2001;46(2):111-9.
4. Huang ZL, Zhou Y, Xiao B, et al. [Proteomic screening of postsynaptic density proteins related with temporal lobe epilepsy]. Zhonghua Yi Xue Za Zhi. 2008;88(45):3205-9.
5. Shu Y, Zhu C, Zeng M, et al. The protective effect of carbenoxolone on gap junction damage in the hippocampal CA1 area of a temporal lobe epilepsy rat model. Ann Transl Med. 2019;7(22):624.

• I strongly suggest that authors showed a time-course of recorded burst activity, since EEG recordings were monitored acutely and chronically in control e pilocarpine rats, displaying the difference between the acute, latent and chronic phase.

Reply: Thanks for raising this good question. To be honest, we did think about this issue at the moment of drafting this paper. Since the model is very mature in our lab and we are very familiar with EEG manifestations and animal behaviors, EEG is not our focus question. We didn't do EEG monitoring electronically, which increased the difficulty of EEG data analysis. That's why we didn't show the time-courses of recorded burst activity in the whole draft. As advised, we added some EEG examples in the part 2 and part 3 of experiments, showing the differences between TLE-SRS

and TLE-NSRS (Fig 1), as well as the differences between groups of TLE-SRS animals pretreated with saline, coc-0.3 ug and pac-1.0 ug (Fig 9). As a result, all the serial number of other pictures were accordingly changed (also marked with Red Color). We also added more explanations in the text to make EEG results clearer (See Page 16 Line 330-333, Page 21, Line 456, 458).

• In the results section, it is reported that the number of animals showing spontaneous seizures after drug injection, out of 20 rats per group. However, in figure 7 the graph depicts a "spontaneous seizure rate" that does not correspond to the number of animals per group indicated and it is not described how it is calculated. The authors should clarify this point.
Reply: Thanks very much for pointing out this obvious mistake for us. Actually, the illustration in the text was correct. However, the figure legend was wrongly explained. We have already modified it as advised (See page 35, line 828-832). Fig 8 B represents the data analysis among different groups of β-tubulin expression under immunoelectron microscopy, not animal numbers exhibiting spontaneous seizures. Thanks again for pointing this for us.

• In the discussion, the authors suggest the mechanism implicated in the protection that is hypothesized to be exerted by microtubule-modulating drugs, considering Ca-mediate glutamate transmission. Though, it cannot be denied that GABA transmission plays a role on the genesis and propagation of epileptic activity, and that microtubule could be implicated (see: Nakajima K, Yin X, Takei Y, et al (2012). The authors should address this point in the discussion.
Reply: Thanks for this precious advice. We have modified our text as advised (see Page 25, line 562-568).

• The paper needs a thorough, extensive revision of the language by an English native speaker. Especially, it would be advisable to use coherently the name of the drugs applied in the study, i.e. "paclitaxel" is for half of the paper called "paclitaxol".
Reply: Thanks for your suggestion. We have changed all "paclitaxol" to "paclitaxel", and have already sought help from English editing service as advised (see track changes in the text).