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LETTERS AND COMMENTS

Concerns regarding Baksa B, Kovacs A, Bayasgalan T, Szentesi P, Koseghy A, Szucs P, Balazs P. Characterization of functional subgroups among genetically identified cholinergic neurons in the pedunculopontine nucleus. Cell Molec. Life Sci. 2019-04-02

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Dear Sirs;

While scientists are people too and can make mistakes, the legacy we leave in science needs to be beyond question. The recent article by Baksa et al. describes poorly performed and erroneously interpreted electrophysiological experiments. It is important, in the absence of a sufficiently expert review, to explain the problems to the readers.

1. The cited article by [5] showed that ALL PPN cell types recorded manifested average initial firing frequencies always > 40 Hz when depolarized using square pulses. Obviously, when you average all of the action potentials (APs) elicited across the entire square pulse, the final frequency was reduced, due to a basic mechanism involving the sustained activation of potassium channels by the square steps (something Baksa et al. described in their Fig. 6G). In fact, subsequent studies showed that square pulses activate these potassium channels and peak power spectrum at gamma frequencies are reduced [3], and explain the need to use, instead of steps, ramps to avoid activation of potassium channels by rapid depolarizing steps. In addition, a recent paper published by our group (Urbano et al. [6]) further described step-by-step how the use of either square pulses or ramps achieved lower or higher power spectra values, respectively. That is, all of the recordings in Baksa et al. are contaminated by potassium channel activity, downregulating AP activity. Moreover, the optimum use of current ramps to study robust gamma band oscillations has been linked to the previously described fact that slowly ramp up current levels when inducing locomotion on a treadmill in the decerebrate animal [1]. Another important limitation was related to the fact that authors did not follow the methodology described by [3], and performed all their work in the absence of synaptic blockers. These authors were oblivious of the fact that underlying, uncontrolled, muscarinic modulation of gamma oscillations would reduce peak frequency of gamma oscillations, as demonstrated in [4]. Without synaptic blockers, this residual spontaneous modulation of PPN neurons will opaquely affect the results. These authors also mentioned that in some experiments, they used TTX (1 μM) (to block sodium channels), or $CdCl_2$ (50 μM), to block calcium channels, but the latter concentration is insufficient to block ALL calcium channels, especially those expressed far away from the cell body and located in the dendrites. For example, [2] used 100 μM to effectively block all calcium channels in a properly designed control study. Altogether, a central problem of the Baksa et al.'s paper is their inability to establish a proper current clamp recording configuration, preventing them from injecting current square pulses over 120 pA, as previously described in [5] and [3], in the absence or presence of synaptic blockers, respectively.
2. There are no references to the critical measure of maximum current amplitude used during ramp depolarization in any figure (Figs. 6A, 6D, 6F, 7C). The authors simply stated in "Materials and Methods" that they used a maximum of 800 pA, with a reference to our papers. Furthermore, all ramp-induced recording are presented

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as insets, never illustrating either control or post-ramp membrane potential changes. Authors averaged out all oscillations to calculate mean frequency of oscillations. It is important to emphasize that in our papers, we calculated the maximal frequency of the three highest amplitude oscillations in each ramp. This is essential, since the ramp travels through the window for high threshold calcium channels, usually –30 mV to –10 mV, and will have a lesser effect on high threshold calcium channel-mediated oscillations before the ramp reaches the window and after it exceeds the window. That is why averaging activity before and after the channels are activated provides an underestimated measure of oscillation frequency.

3. The range of resting membrane potentials used by Baksa et al. was 10–30 mV (from − 60 to − 80 mV), which is more hyperpolarized than is required for the activation of high threshold calcium channels. Therefore, their results will have major contamination provided by T-type channel-mediated spikes/currents, which is clearly evident in Fig. 6D (top ramp-induced recording). In conclusion, it is likely that they did not design their intracellular solution to properly control resting potential level, and decided to simply inject low levels of current to slightly depolarize their Vm. To properly study high threshold calcium channels, all of these factors are critical, and while the methodology is described clearly in the literature, it is ignored in Baksa et al.
4. During the double recordings they mentioned on Fig. 7B, no distant dendrites were actually recorded. They clearly described problems recording from dendrites over 25 µm away from the soma, which would fail to detect what [2] described as dendritic calcium flow at locations > 40 um away from the soma [2]; Fig. 4D). Baksa et al. failed to acknowledge the voltage-clamp data presented in [3], describing how to appropriately clamp the soma of PPN neurons, revealing the persistence of significant gamma oscillatory activity using proper clamping, currents which obviously arise in distant dendritic compartments that are always more difficult to record without proper voltage control. Astonishingly, they included the following paragraph on page 14: "However, based on the present data, one cannot exclude that distal dendrites have own oscillatory activity. [..]". Given this significant caveat, the authors, nevertheless, proposed that their data supported the erroneous assessment (in the abstract and several sections of the paper) that oscillations arose only from the soma. Finally, they do not seem to understand the difference between resonance and origin of oscillations. In conclusion, they have never dissected the underlying mechanisms of such oscillations using local dendritic recordings.

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