

Peer Review

Review of: "Cholinergic Signaling Differentially Regulates Song Premotor Circuits to Stabilise Songs in Songbirds"

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Many thanks to the authors (Xu et al.) for sharing this informative work with the public. In this paper, they mainly used patch clamp recording and neuro-pharmacological methods to study how cholinergic modulations shape the synaptic inputs received by projection neurons in RA (RAPN), a major premotor nucleus crucial for driving the song. The results are particularly valuable for those considering how animal behaviors are modulated. However, I must say that the reliability of the data interpretation is weakened by some technical concerns. These are as follows:

1. In figures 1BCF and 2BCF, the cumulative curves of mini amplitude show an increase, while the corresponding bar plots show the opposite. Something must be wrong. Please double-check.
2. In all figures, since most comparisons are pairwise, scattered individual data points with lines connecting paired ones (e.g., pre- and post-drugs) should be plotted instead of bar plots.
3. Brain sections were made coronally as described in the methods, but sagittal views are shown in figures 1 and 2. Please clarify exactly how the slices were made.
4. Please double-check the composition of the slicing sucrose solution. The listed recipe (62.5mM NaCl, 5mM KCl, 28 mM NaHCO₃, 248 mM sucrose, 1.3mM MgSO₄·7H₂O, 10mM glucose, and 1.26mM NaH₂PO₄·H₂O) may yield an osmolarity of over 400 instead of 330–340.
5. Additional description of the patch clamp recordings is necessary. For example, what are the holding potentials for recording the minis and evoked epscs? What is the strength of extracellular stimulation (figures 3, 4, and 5), and how is it chosen? Is the access resistance monitored all the time, and how is it compensated?
6. It's not clear if the HVC–Ra and Lman–Ra pathways are well separated in the preparation. An additional test to clarify the specificity of extracellular stimulation is by pairing stimuli in different pathways and examining the PPR to see if there is short-term interaction across different stimuli.
7. In figure S1, it's better to calculate the acoustic properties of each syllable and compare syllable-wise rather than analyzing the whole song motifs.
8. In figure 6D, it's a bit confusing that the similarity to the bird's own song is so variable even among those “Pre” groups, which are supposed to be without any treatment. The elevated similarity scores by carbacol and OM are at the same level as the controls of the last two experiments. How to justify this?

Other points:

1. Is the cholinergic modulation of synaptic transmission and song output reversible? No washout experiments were shown.
2. What is the hypothesized physiological condition when acetylcholine can be released in Ra? This is important when thinking about the experiment shown in figure 6. Is Ach released when birds are singing? It's better to discuss this more.
3. It's not mentioned what anesthetic was used in the study.

Declarations

Potential competing interests: No potential competing interests to declare.