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

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Potential competing interests: No potential competing interests to declare.

Xu and colleagues provide evidence of pathway-selective cholinergic neuromodulation of synaptic transmission in the songbird motor cortical analog RA. In detail, they find that in male zebra finches, muscarinic agonism effectively reduces excitatory transmission at HVC-RAPNs synapses, while nicotinic signaling reduces LMAN-RAPNs neurotransmission. In contrast, the non-singing female neural circuitry is sensitive to muscarinic signaling only, and specifically at the LMAN-RAPN locus, contrary to what is observed in the male. This marked difference is enticing and provides an elegant comparison between the “base” and the “song-permissive” states of the same neural circuit. The authors then examine the acute effects of direct cholinergic agonism in RA and claim that muscarinic, but not nicotinic, stimulation results in a stabilization of the adult directed song. They conclude the manuscript hypothesizing a modulatory effect of cholinergic signaling in the different conditions and districts examined.

While the electrophysiological experiments seem well conducted, several aspects of the behavioral study, as well as the general interpretation of the results, are problematic.

Major points:

1. The authors find that the neuromodulatory effect of cholinergic agonism reduces neurotransmission through a presynaptic mechanism, yet throughout the paper, and especially in the discussion, they talk about cholinergic receptor expression at the postsynaptic level in RAPNs. They could have easily spun the whole story towards presynaptic regulation of neurotransmission[1-3]. This would actually be even more interesting, especially in light of the reported segregation of RA afferent excitatory neurotransmission (AMPAR- vs. NMDAR-mediated at HVC vs. LMAN terminals, respectively). Instead, the authors discuss work reporting postsynaptic effects (excitability modulation) that can’t account for the observed synaptic effects, and no mechanism is offered for how the presynaptic neurotransmission would be reduced by postsynaptic cholinergic agonism.
2. The behavioral effects are at best dubious: female directed song has been reported by multiple investigators to be even more reliable and stereotyped than undirected song [4,5]. Yet, the measured self-similarity seems to be unrealistically low specifically for the control groups of the CAR and OM groups, which are the ones showing the significant effect. However, upon CAR and OM, the self-similarity scores are at the same level for both conditions (pre and post) in the DMPP and PBS groups. This undermines the reliability of the behavioral result.
3. Even if point 2 was addressed, the explanation of why HVC-RAPNs specific muscarinic agonism would reduce variability is not consistent with the existing literature. HVC projections to RA are reportedly providing timing and/or

gesture representation to RA, while the AFP through LMAN provides variability to the syllables[6-8]. The authors instead speculate a S/N reduction by muscarinic agonism that would render the HVC afference more refined, but they don't provide evidence for it. The absence of effects by the nicotinic agonist, despite previous reports indicating that suppressing LMAN-RAPN transmission promotes stereotypy, is counterintuitive and should be at least discussed.

4. Figure 7 is purely speculative, appears hand-drawn, and should be used in the context of a review, not a paper.

Minor points:

1. The voltage clamp experiments are conducted with a K+-based internal solution; therefore, the authors can't assume effective distal clamping of the Vm. The experiments would have been better conducted with Cs2+ in the internal solution, and potentially with TEA.
2. The histograms used to represent the data are not the best plot choice, especially given that all data is compared in paired statistics. The data should be shown as box + scatter plots, and pre-post for each cell should be connected by a line, so that readers can appreciate the extent of coherence in the direction of the change exerted by the pharmacological agents. Alternatively, the authors should normalize each cell's response to the baseline state and plot just the % change.
3. When recording mEPSCs, the decrease in amplitude may fully explain the decrease in frequency, as events that would have been barely passing the threshold for consideration would now fall under it and not get counted. The authors should (as per many of the papers they cited) test the synaptic transmission with 10Hz stimulation trains to confirm the presynaptic origin of the cholinergic-mediated reduction of transmission. The fact that they find the PPR to be affected by the agonism in later figures partly renders this unnecessary, so it's a minor point, but in general, we shouldn't assume it's a presynaptic effect just because of the reduction in frequency.
4. It would have been nice to have the PPR plotted also for the cases in which the amplitude of evoked EPSCs didn't change upon pharmacological application.
5. The authors should avoid plotting data related to multiple pharmacological conditions on the same plot if they don't intend to compare the data with appropriate statistical tests (e.g., Fig. S1, Fig. 6 require 2-way ANOVA tests; it's not correct to compare each group separately from the others with paired t-tests).
6. The PPR is claimed to be assessed at an inter-pulse interval of 50ms, but the scalebars in figs 3, 4, and 5 indicate it's 25ms. Either the PPR is 25ms or the scalebar is wrong.
7. The analysis of spectral characteristics in Suppl. Fig. 1 makes little sense, as measuring those parameters across the entire motif is pointless. The analysis should be conducted by syllable.
8. Multiple references are not correct, pointing to the wrong papers (e.g., Introduction: 6 is about Bengalese finches changing their syntactical transitions in different contexts, not about template-guided song learning. The authors should have cited any review by Konishi, Mooney, Brainard, Fee, Long, Roberts, etc. Discussion: 23 and 24 don't report about amphibians, 33 is about PD-like states and not motor skill acquisition, 26 is about metaplasticity in the hippocampus; the authors probably meant 27; etc.).
9. Grammatical and syntactical errors are frequent and impair fluent reading of the manuscript. I would advise the authors to collaborate with an English mother-tongue researcher or editor to help with the writing (I'm Italian; I

understand the struggle too well, but our lack of familiarity with English ultimately impairs our scientific communication).

Altogether, while the topic is potentially interesting and the electrophysiological investigation is appealing, the conclusions are not supported by the data, and in particular, the behavioral data from this manuscript should be considered with extreme caution.

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