# Cholinergic signaling differentially regulates song premotor circuits to stabilize songs in songbirds

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**Running Title: Cholinergic Signaling Differentially Regulates Birdsong**

## SUMMARY

Cholinergic modulation plays an important role in motor skill learning, including vocal learning. In songbirds, song premotor nucleus RA simultaneously receives inputs from song nuclei HVC and LMAN, and then its projection neurons (RAPNs) generate song motor control output. Using electrophysiological and pharmacological methods, we found that cholinergic signaling can enhance song stability by reducing HVC-RAPN excitatory synaptic transmission in adult male zebra finches, mediated by mAChRs. Although nAChRs are not effective overall, cholinergic signaling can also decrease LMAN-RAPN excitatory synaptic transmission induced by electrical stimulation via nAChRs, suggesting the potential role of cholinergic regulation in song behavior through LMAN-RA pathway. On the contrary, in adult female zebra finches, only LMAN-RAPN synaptic transmission was reduced by cholinergic signaling via mAChRs. The role of differential cholinergic regulation of song premotor circuits in songbirds' singing provides insights into the neural processes of motor skill learning.

**Key words:** cholinergic modulation, mAChRs, nAChRs, songbirds, song control pathways, synaptic transmission, song behavior, sex differences

## In brief

Xu et al. reveal a mechanism of cholinergic modulation on songbird singing. They find that cholinergic signaling differentially regulates HVC-RAPN and LMAN-RAPN synaptic transmission via mAChRs and nAChRs respectively to stabilize songs in male zebra finches, while in females, only LMAN-RAPN synaptic transmission is regulated by cholinergic signaling via mAChRs.

## Highlights

- Cholinergic signaling regulates HVC-RA pathway of male songbirds by mAChRs
- Cholinergic signaling regulates LMAN-RA pathway of male songbirds by nAChRs
- Cholinergic signaling stabilizes birdsongs in males by mAChRs rather than nAChRs
- Cholinergic signaling merely regulates LMAN-RA pathway in females by mAChRs

## INTRODUCTION

The learning and long-term retention of motor skills play a fundamental role throughout an individual's lifespan, such as handwriting, cycling, piano playing, dancing, and effective speech communication.[1] These are typically acquired gradually through multiple learning sessions until performance reaches stability,[2] within which cholinergic signaling assumes a pivotal role.[3,4] However, the process and mechanism of cholinergic regulation on motor skill learning remain unclear.

Similar to human speech, songbird singing is a rare vocal learning behavior in animals and also a form of complex motor skill learning.[5] Juvenile songbirds optimize the matching degree between their own singing and the instructional song template through repetitive practice and self-correction relying on auditory feedback, and their songs progressively stabilize during the course of attaining adulthood.[6] Two well-defined neural pathways in songbird brain, namely vocal motor pathway (VMP) responsible for vocalization and anterior forebrain pathway (AFP) responsible for juvenile song learning and adult song plasticity, coordinately control singing.[7,8] VMP is composed of the song premotor nuclei HVC (proper name) and the robust nucleus of the arcopallium (RA), as well as brainstem motor nucleus; AFP represents a neural circuit, where its output nucleus the lateral magnocellular nucleus of the anterior nidopallium (LMAN) projects to RA of VMP, while its input nucleus area X receives the afferent from HVC of VMP.[9]

As an analogous structure of human laryngeal motor cortex, RA serves as the convergence nucleus within song premotor circuits, and concurrently receives glutamatergic projections from its upstream motor nucleus HVC and LMAN of AFP (Figure 1A).[10,11] Meanwhile, RA receives cholinergic projections from the ventral pallidum (VP) in basal forebrain.[12,13] During the critical period of song learning, an elevation of acetylcholine (ACh) concentration within RA was observed in male zebra finches.[14] The combined infusion of muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) antagonists into RA leads to abnormal song development during the critical period.[15] RA consists of two types of neurons, including RA interneurons and RA projection neurons (RAPNs) that encode the acoustic properties of song syllables and input the information to brainstem motor nucleus.[16-18] Our previous work showed that cholinergic signaling primarily

modulates RAPNs' electrophysiological activities in adult male zebra finches via mAChRs, but not nAChRs.[19] These studies indicate that RA is a crucial target of cholinergic modulation. Nonetheless, the essential mechanism by which cholinergic signaling governs RA to impact song production remains unknown. To clarify this issue, it is indispensable to understand the mechanism through which cholinergic signaling regulates the song premotor circuits with RA as the core.

In this study, we initially employed in vitro patch-clamp whole-cell recording combined with pharmacological approaches to separately examine the roles of two cholinergic receptors, mAChRs and nAChRs, in cholinergic modulation of RAPNs' excitatory synaptic afferents in adult male zebra finches. Given that the song premotor signals input to RA are respectively derived from HVC and LMAN, we conducted a further investigation into the influences of different cholinergic receptors on HVC-RAPN and LMAN-RAPN excitatory synaptic transmission. Subsequently, we used in vivo targeted pharmacological manipulation combined with song analysis to detect the effects of different cholinergic receptors within RA on birdsongs. Moreover, considering that zebra finches manifest typical sexual dimorphism, which only males sing and females never do,[10] we also explored the cholinergic modulation of the song premotor circuits similar to that of males in adult female zebra finches. Overall, our findings delineate a gender-differentiated mechanism by which cholinergic signaling differentially regulates song premotor circuits via mAChRs and nAChRs, thereby potentiating song behavior. Finally, we put forward a rational hypothesis concerning cholinergic modulation of song learning process.

## RESULTS

### Cholinergic signaling reduces RAPNs' excitatory synaptic afferents in adult male and female zebra finches via mAChRs rather than nAChRs

To investigate the cholinergic modulation of RAPNs' excitatory synaptic afferents in adult zebra finches, we first respectively recorded the influences of cholinergic receptor agonists and antagonists on RAPNs' mEPSCs. The results showed that the non-selective cholinergic receptor agonist carbachol (CAR) significantly decreased the frequency and amplitude of RAPNs' mEPSCs in both males (Figure 1B) and females (Figure 2B), indicating that CAR can reduce RAPNs' excitatory synaptic afferents in adult male and female zebra finches through both pre-

and postsynaptic mechanisms.

The mAChR agonist Oxotremorine M iodide (OM) mimicked the effects of CAR, which also significantly reduced the frequency and amplitude of RAPNs' mEPSCs in both males (Figure 1C) and females (Figure 2C). However, the nAChR agonist DMPP had no effect on the frequency and amplitude of RAPNs' mEPSCs in both males (Figure 1D).and females (Figure 2D). If CAR and the mAChR antagonist atropine (ATR) were simultaneously added, the effect of CAR was blocked by ATR, and no significant changes were observed in the frequency and amplitude of RAPNs' mEPSCs in both males (Figure 1E) and females (Figure 2E). When CAR and the nAChR antagonist mecamylamine (MEC) were co-administered, the effect of CAR was not blocked by MEC, and the frequency and amplitude of RAPNs' mEPSCs remained significantly decreased in both males (Figure 1F) and females (Figure 2F). This finding suggests that cholinergic signaling reduces RAPNs' excitatory synaptic afferents in adult male and female zebra finches via mAChRs but not nAChRs.

**Cholinergic signaling reduces HVC-RAPN excitatory synaptic transmission in adult male zebra finches via mAChRs rather than nAChRs**

HVC-RA pathway is a crucial excitatory synaptic afferent of RA. Our further results showed that CAR significantly reduced the amplitude of RAPNs' evoked EPSCs (eEPSCs) induced by stimulating HVC-RA projection fibers in adult males (Figure 3B), indicating that CAR can prominently decrease HVC-RAPN excitatory synaptic transmission. Meanwhile, the effect of CAR was accompanied by a significant increase in paired-pulse facilitation ratio (PPR) (Figure 3B), suggesting that presynaptic mechanism was involved in the role of CAR on HVC-RAPN excitatory synaptic transmission.

OM mimicked the effect of CAR, which likewise significantly reduced HVC-RAPN eEPSCs' amplitude (Figure 3C) and caused a significant elevation in PPR (Figure 3C). However, DMPP had no effect on HVC-RAPN eEPSCs' amplitude (Figure 3D). If CAR and ATR were concurrently administered, the inhibitory action of CAR on HVC-RAPN eEPSCs' amplitude was obstructed by ATR (Figure 3E). Nevertheless, when CAR and MEC were concurrently administered, MEC failed to neutralize the effect of CAR (Figure 3F). These results suggest that cholinergic signaling reduces HVC-RAPN excitatory synaptic transmission in adult male zebra finches via mAChRs but not nAChRs.

**Cholinergic signaling reduces LMAN-RAPN excitatory synaptic transmission in adult male zebra finches via nAChRs rather than mAChRs**

LMAN-RA pathway is another important excitatory synaptic afferent of RA. CAR significantly decreased the amplitude of RAPNs' eEPSCs elicited by stimulating LMAN-RA projection fibers in adult males (Figure 4B), indicating that CAR can markedly reduce LMAN-RAPN excitatory synaptic transmission. During the CAR effect, PPR was significantly increased (Figure 4B), suggesting that presynaptic mechanism was involved in the role of CAR on LMAN-RAPN excitatory synaptic transmission.

OM exerted no influence on LMAN-RAPN eEPSCs' amplitude (Figure 4C). However, DMPP simulated the effect of CAR, significantly reducing LMAN-RAPN eEPSCs' amplitude (Figure 4D), and simultaneously causing a significant increase in PPR (Figure 4D). When CAR and ATR were applied together, the reduction effect of CAR on LMAN-RAPN eEPSCs' amplitude was not blocked by ATR (Figure 4E). If CAR and MEC were applied simultaneously, the effect of CAR was blocked by MEC (Figure 4F). These results demonstrate that cholinergic signaling reduces LMAN-RAPN excitatory synaptic transmission in adult male zebra finches via nAChRs but not mAChRs. Namely, receptor mechanisms of cholinergic modulation on the excitatory synaptic transmission of the two song premotor pathways, LMAN-RA and HVC-RA, in males are different.

**Cholinergic signaling has no effect on HVC-RAPN excitatory synaptic transmission in adult female zebra finches**

In contrast to the findings in males, CAR exerted no impact on HVC-RAPN eEPSCs' amplitude in adult females (Figure 5B). Additionally, neither DMPP (Figure 5C) nor OM (Figure 5D) had any effect on HVC-RAPN eEPSCs' amplitude. This postulates that HVC-RAPN excitatory synaptic transmission in adult female zebra finches is not regulated by cholinergic signaling.

**Cholinergic signaling reduces LMAN-RAPN excitatory synaptic transmission in adult female zebra finches via mAChRs rather than nAChRs**

Similar to males, CAR significantly reduced LMAN-RAPN eEPSCs' amplitude in adult females (Figure 5E), and led to a significant increase in PPR (Figure 5E),

suggesting that there are also presynaptic mechanisms involved in the CAR modulation of LMAN-RAPN excitatory synaptic transmission in females.

OM mimicked the reduction effect of CAR on LMAN-RAPN eEPSCs' amplitude in females (Figure 5F), accompanied by a significant increase in PPR (Figure 5F). Whereas, DMPP had no influence on LMAN-RAPN eEPSCs' amplitude in females (Figure 5G). Simultaneously applying CAR and ATR, the effect of CAR was blocked by ATR (Figure 5H). While simultaneously applying CAR and MEC, the effect of CAR was not blocked by MEC (Figure 5I). These results indicate that, contrary to the receptor mechanism of cholinergic modulation on LMAN-RAPN excitatory synaptic transmission in males, cholinergic signaling reduces LMAN-RAPN excitatory synaptic transmission in adult female zebra finches via mAChRs but not nAChRs.

**Cholinergic signaling within RA enhances song stability of adult male zebra finches via mAChRs rather than nAChRs**

To further validate the impact of cholinergic signaling within RA on adult song behavior and its receptor mechanisms, the results of behavioral experiments combined with an in vivo pharmacological method (Figure 6A and B) showed that CAR microinjection onto RA significantly elevated the similarity to own song of adult males (Figure 6C and D) and reduced the song entropy (Figure 6C and E). OM microinjection onto RA exhibited a comparable effect to CAR, which also increased song similarity (Figure 6C and D) and decreased song entropy (Figure 6C and E), whereas DMPP microinjection had no effect on song similarity (Figure 6C and D) and entropy (Figure 6C and E). For comparison, the results of phosphate buffered saline (PBS) microinjection showed that there was no obvious change in song similarity (Figure 6C and D) and entropy (Figure 6C and E), thereby ruling out the influence brought by the microinjection operation per se. In addition, other song acoustic characteristics were not notably influenced by the diverse drug microinjections (Figure S1). These results manifest that cholinergic signaling within RA enhances song stability of adult male zebra finches via mAChRs but not nAChRs.

## DISCUSSION

To illuminate cholinergic modulation of songbirds' song behavior, it is

233 indispensable to elucidate the cholinergic regulatory mechanism at the neural pathway
234 level. We discover a mechanism by which cholinergic signaling separately regulates
235 song premotor circuits via different receptors, thereby enhancing song stability of
236 adult males. Moreover, a similar mechanism exists in the same circuits of adult
237 females. Furthermore, we postulate a hypothesis concerning cholinergic regulatory
238 process of song learning in juveniles and song maintenance in adults. From a broader
239 perspective, our findings provide significant insights into cholinergic regulation of
240 motor skill learning.
241
242 **Songbirds are an excellent animal model for revealing cholinergic modulation of**
243 **motor skill learning**
244 Central cholinergic system exists in the brains of fish,[20-22] amphibians,[23,24]
245 reptiles,[23,24] mammals,[25-28] and birds,[29-32] which mainly participates in various higher
246 neural activities such as motor skill acquisition,[3,33] learning and memory,[34,35] sleep
247 and wakefulness,[36,37] and attention maintenance.[38,39] ACh exerts different effects by
248 binding to two different types of receptors, mAChRs (G-protein coupled receptors)
249 and nAChRs (ligand-gated ion channel receptors). Both receptors are expressed in all
250 layers of the vertebrate cerebral cortex,[20,28,40] with different expression patterns among
251 different layers and cell types.[41] For example, it has been reported that nAChRs is
252 mainly expressed on interneurons in the prefrontal cortical layer 2/3 of rats, while
253 only a small portion of pyramidal neurons express nAChRs.[42] It was found that there
254 are regional and interlayer differences in the expression of the two main cortical
255 mAChR subtypes (M1 and M2) in the prefrontal cortex of rhesus monkeys.[43]
256 The construction of avian subcortical cholinergic system is analogous to that of
257 mammals. Research has confirmed that the cholinergic system of pigeon in basal
258 forebrain, epithalamus, isthmus, and hindbrain closely resembles that of reptiles and
259 mammals.[29] Further research indicates that the basal telencephalic cholinergic system
260 in pigeons and budgerigars is anatomically very similar to the cholinergic basal
261 nuclear system in mammals, particularly in terms of their projection patterns in
262 cerebral cortex or pallium.[32,44] Although the neurons in avian pallium are not
263 hierarchically organized like those in mammalian cerebral cortex, cholinergic receptor
264 distribution in avian brain also shows regional specificity.[30,31]
265 In songbirds, both male and female zebra finches have cholinergic projection
266 fibers in song nuclei and auditory nuclei.[45,46] Studies have shown that HVC and RA

are rich in acetylcholinesterase, suggesting a strong cholinergic dominance over these two nuclei.[45-47] The cholinergic innervation of HVC and RA originates from VP, which is analogous to the mammalian basal forebrain cholinergic system, specifically the nucleus basalis of Meynert.[12]

Evidence suggests that mAChRs are expressed within song nuclei,[48,49] and nAChRs are also present in multiple song nuclei.[50] Asogwa et al. cloned four out of the five mammalian mAChR subunits (Chrm2-5) in male zebra finches. They found that, at each developmental stage, the expression of excitatory subunits (chrm3 and chrm5) consistently exhibits lower levels compared to inhibitory subunits (chrm2 and chrm4) within most song nuclei, including RA.[51] Recently, all 15 types of nAChR subunits in male zebra finch brain were cloned. It was confirmed that most nAChR subunits (except for ChrnA1, A6, A9, and A10) are expressed in song related pathways during the critical period of song learning, while only 6 nAChR subunits (ChrnA2-5, A7, and B2) are expressed in adulthood.[52] This suggests that the expression of most nAChR subunits undergoes continuous changes throughout song formation process in various song nuclei, and the expression types of nAChR subunits in adulthood are much fewer than those during song learning.

Central cholinergic system plays a crucial role in the regulation of motor skill learning.[53-55] However, the precise mechanisms through which this system governs such learning remain elusive, and elucidating its role poses significant challenges. Songbird's song learning is a complex form of sensory-motor learning, which is an extremely rare vocal learning behavior similar to human language learning.[5] Pharmacological and behavioral research showed that subcutaneous administration of mid (0.18mg/kg) and high (0.54mg/kg) dose of the nAChR agonist nicotine can elicit sensitized responses of song production and locomotor activity in adult male zebra finches.[56] Continuous nicotine administration for 7 days can alter the tempo and rhythm of adult crystallized songs, and this effect may persist for an extended period (over 2 months) after discontinuation of nicotine administration.[57] These findings indicate that central cholinergic system likely plays a significant role in the vocalization and song learning processes of songbirds. Therefore, songbirds could serve as an excellent research model for in-depth exploration of cholinergic regulation on complex motor control and learning mechanisms, including human speech.

**A mechanism by which cholinergic signaling separately regulates song premotor**

**circuits of male songbirds through different receptors thereby promoting song stability**

The electrophysiological activities of HVC and RA are associated with the neural encoding of song motor. The motor control signals generated by HVC require processing through RA in order to ultimately produce appropriate song motor encoding for coordinated activation of syringeal and respiratory muscles.[58,59] Shea and Margoliash found that cholinergic signaling attenuates the auditory responses of HVC and RA to bird's own song in adult male zebra finches.[60] Furthermore, distinct HVC projection neurons (HVC-to-RA and HVC-to-area X projection neurons) exhibit differential responses to cholinergic signaling, and this diversity of response may facilitate the coordination of cholinergic regulation between HVC-RA motor command transmission and the reception of HVC motor signals by AFP.[61] Jaffe and Brainard found that dialyzing CAR into HVC increases HVC neuron activities, resulting in an elevation of song pitch, amplitude, tempo and stereotypy to levels approaching those of direct courtship songs in adult male Bengalese finches, which can be weakened by blocking mAChRs.[62]

An early study revealed a notable elevation in ACh levels within HVC, LMAN and RA of zebra finches during the critical period for song acquisition, followed by a gradual decline as the birds approach adulthood.[14] ACh transient increase within RA during critical period is associated with heightened acetyltransferase (ChAT) activity.[63] Additionally, biochemical research has shown that during the critical period of song learning in zebra finches, CAR significantly increases the phosphoinositide turnover within the synaptoneurosomes of RA neurons.[64] Through intracellular electrophysiological recordings in brain slices, Salgado-Commissariat et al. discovered that nicotine enhances RA neuron excitability in adult male zebra finches, without distinguishing between the types of RA neurons (projection neurons or interneurons). Furthermore, by activating different nAChR subtypes, they demonstrated that tetanic stimulation induces bidirectional synaptic plasticity (long-term potentiation and long-term depression) in LMAN-RA pathway.[65]

RAPNs are homologous to layer 5 pyramidal neurons in mammalian motor cortex, and their activities govern song production.[66,67] Our previous results obtained from brain slice whole-cell current-clamp recordings indicate that cholinergic signaling can induce a hyperpolarization of membrane potential, an increase in afterhyperpolarization potential and membrane conductance, and a reduction in action

potential firing in RAPNs of adult male zebra finches via mAChRs rather than nAChRs.[19,68] This suggests that under physiological conditions, cholinergic signaling may influence RAPN activities by altering their intrinsic membrane properties, thereby regulating song behavior. In present study, the results of brain slice whole-cell voltage-clamp recordings showed that cholinergic signaling significantly reduces RAPNs' mEPSCs in adult male zebra finches through mAChRs rather than nAChRs, indicating that RAPNs' excitatory synaptic afferents in adulthood is regulated by cholinergic signaling, and mAChR mediated action remains predominant. Cholinergic regulation on RAPNs' intrinsic membrane properties and excitatory synaptic afferents is predominantly mediated by the inhibitory effect of mAChRs to reduce excitability, which is consistent with the circumstance that the inhibitory mAChR subunits are expressed significantly more than the constitutively low-expressed excitatory mAChR subunits within RA at all development stages.[51]

In mammals, similar to our results, ACh local application inhibits the excitability of layer 5 pyramidal neurons in rat prefrontal cortex, somatosensory cortex, and visual cortex.[69] Another study has confirmed that ACh induces membrane potential hyperpolarization and enhances membrane conductance of layer 5 pyramidal neurons in rat neocortex via mAChRs, thereby exerting an inhibitory action.[70] Additionally, it has been reported that CAR reduces glutamatergic excitatory synaptic transmission of layer 5 pyramidal neurons in rat sensory cortex, and this effect is blocked by mAChR antagonists.[71] In fact, the impacts of cholinergic signaling on neurons in diverse cortical areas and different layers are not homogeneous. A study has found that CAR increases the firing of rat neocortical neurons through different mAChR subunits, but reduces their synaptic transmission.[72] Generally, ACh can exert an excitatory action on neurons at the deepest layer (layer 6) of mouse primary cortex, including primary somatosensory cortex, primary motor cortex and combined medial prefrontal cortex. It has been disclosed through pharmacological methods that nAChRs and mAChRs jointly contribute to mediating the ACh's action on the neurons in layer 6 of primary motor cortex. However, the neurons in layer 6 of combined medial prefrontal cortex display a stronger response to ACh, and the effect is mainly mediated by nAChRs. The response of layer 6 neurons in primary somatosensory cortex to ACh is relatively weak, and the receptor mechanism of neurons therein affected by ACh is inconsistent.[26] By comparing the aforementioned studies on mammals with our experimental results, it can be noted that the cholinergic regulation of song premotor

brain regions in songbirds exhibits both similarities to that of cerebral cortex in mammals and interspecific dissimilarities or its functional specificity.

It is worth noting that the pharmacological research on layer 6A pyramidal cell types of rat somatosensory cortex in recent years has revealed that low concentration (30 μM) ACh can induce hyperpolarization of corticocortical pyramidal cells via M4 and M1 mAChRs, while depolarizing corticothalamic pyramidal cells. However, under the influence of high concentration (1 mM) ACh, corticothalamic pyramidal cells can be depolarized via α4β2 nAChRs, while the membrane potential of corticocortical pyramidal cells is unaffected. Nevertheless, mEPSCs' frequency of corticocortical pyramidal cells is reduced, whereas that of corticothalamic pyramidal cells is increased. Additionally, glutamate synaptic transmission in corticocortical pyramidal cells is inhibited via M4 mAChRs, but in the corticothalamic pyramidal cells, it is enhanced via α4β2 nAChRs. This study indicates a potential mechanism through which ACh might separately regulate diverse projection neurons in the same cortical region via both nAChRs and mAChRs, thus achieving the coordination among synaptic transmission functions.[41] However, within the song premotor nucleus RA of songbirds, there is merely one type of projection neurons (projecting to brainstem motor nucleus). The integration of song premotor signals from HVC-RA and LMAN-RA pathways within RAPNs is the critical determinant in generating the encoded output for controlling song production.[73,74] The more crucial mechanism underlying cholinergic modulation of song behavior might lie in how to achieve the discrete regulation of HVC-RA pathway and LMAN-RA pathway, concomitant with the reciprocal coordination and collaboration in the regulation between the signal transmissions of the two pathways. Our present results revealed that cholinergic signaling markedly decreases glutamatergic HVC-RAPN eEPSCs' amplitude via mAChRs in adult male zebra finches. On the contrary, the reduction of glutamatergic LMAN-RAPN eEPSCs' amplitude caused by cholinergic signaling was mediated by nAChRs in adult males. Meanwhile, cholinergic regulation of both HVC-RAPN and LMAN-RAPN synaptic transmission is accompanied by alterations in PPR (an electrophysiological indicator suggesting presynaptic mechanisms[75]). Our discoveries demonstrate a mechanism by which cholinergic signaling achieves the distinct regulation of song premotor circuits through two receptors, nAChRs and mAChRs. Recently, it has also been found in adult mice that cholinergic signaling can differentially regulate the glutamatergic synaptic transmission of the projections from

prelimbic cortex and thalamus to Basolateral Amygdala pyramidal neurons, respectively, through different receptors.[76] The disclosure of the differential regulatory mechanism of cholinergic receptors further underpins the crucial role of cholinergic regulation adapted to specific functions in relevant brain regions.

Most tellingly, our further behavioral research combined with in vivo targeted pharmacological manipulation indicated that CAR microinjection onto RA significantly enhances song stability of adult male zebra finches. Furthermore, microinjection of mAChRs agonist has the same effect as CAR, whereas microinjection of nAChRs agonist has no effect. The result is consistent with the finding of Jaffe and Brainard in adult male Bengalese finches that dialysis infusion of CAR into HVC affects singing, both increasing song stability.[62] Concurrently, the result indicates that cholinergic signaling within RA governs song behavior in adulthood predominantly via mAChRs rather than nAChRs. This receptor mechanism is consistent with that of cholinergic signaling regulating RAPNs' mEPSCs and intrinsic membrane properties in adult males, all through mAChRs but not nAChRs.[19] Taken together, given that RAPNs are marked by the electrophysiological characteristic of high excitability,[77,78] cholinergic signaling can diminish the glutamatergic excitatory synaptic transmission of song premotor pathways in adult males via mAChRs, thus appropriately lowering the high excitability of RAPNs and potentially enhancing their information integration capacity by improving signal-to-noise ratio through this mechanism, ultimately promoting song stability. We recorded that RAPNs' excitability in 26-day-old fledglings (at subsong stage) is conspicuously higher than that in adults at song crystallization stage (data not shown), which is in accordance with our anticipation that the decrease in RAPNs' excitability is associated with the enhancement of song stability. Moreover, evidence indicates that apart from cholinergic signaling, RA is also controlled by a multitude of other neurotransmitters, such as monoamine neurotransmitters.[79-83] Cholinergic signaling could merely be one constituent in the modulation of the chemical "cocktail". Meanwhile, RAPNs' inhibitory synaptic inputs from the GABAergic interneuron network would exert an important balancing effect.[84]

Another investigation has validated that cholinergic signaling within RA also has a substantial impact on song learning during developmental stage. Puzerey et al. employed in vivo reverse microdialysis technique to administer nAChR and mAChR antagonists in juvenile zebra finch RA for the purpose of chronically blocking

437 cholinergic signaling in RA during song learning. After several weeks, it was
438 discovered that singing quantity declines, and song learning process becomes
439 disordered, such as excessive increase in song variability, abnormal acoustic features,
440 and a decrease in similarity to tutor song.[15] Furthermore, throughout the process of
441 development and song acquisition, the synaptic density and amount of HVC-RA
442 projection fibers increase conspicuously, yet the synaptic density and amount of
443 LMAN-RA projection fibers diminish markedly.[11,85] Although LMAN-RA pathway
444 assumes a pivotal role in song learning[86,87] and provides motor correction signals for
445 HVC-RA pathway,[59,88,89] and the lesion of LMAN in juvenile zebra finches can
446 disrupt song acquisition, the lesion of LMAN in adulthood has no impact on the
447 already stable songs.[90,91] Consequently, based on the findings of predecessors and
448 ours, it is reasonable to believe that during the song learning period of juvenile zebra
449 finches, the mutual coordination and collaboration between the cholinergic regulation
450 of LMAN-RA pathway mediated by nAChRs and that of HVC-RA pathway mediated
451 by mAChRs exert a significantly facilitating effect on the process of song learning.
452 However, during the process of song gradually stabilizing, with the weakening of
453 LMAN-RA pathway and the strengthening of HVC-RA pathway, the cholinergic
454 modulation of LMAN-RA pathway mediated by nAChRs also weakens accordingly.
455 Therefore, the reinforcing effect of cholinergic signaling on the stability of adult
456 birdsongs is mainly regulated by HVC-RA pathway mediated via mAChRs. Herein,
457 we propose a mechanism hypothesis that cholinergic signaling influences song
458 production by regulating song premotor circuits (Figure 7A), and we are currently
459 undertaking a study on cholinergic modulation of song premotor circuits during song
460 learning period of juvenile zebra finches. The finding of Asogwa et al. that the
461 expression types of nAChR subunits in adulthood within song related pathways,
462 including LMAN-RA pathway, are much fewer than those in critical period of song
463 learning offers support for our hypothesis.[52]

464 Although cholinergic modulation of LMAN-RA pathway mediated by nAChRs
465 turns quiescent or latent in adulthood, our experimental observations still imply that in
466 the context of electrical stimulation of LMAN-RA projection fibers, cholinergic
467 signaling can give rise to a decline in LMAN-RAPN excitatory synaptic transmission
468 in adult males via nAChRs, suggesting that this pathway is likely to undergo adaptive
469 modifications under the elicitation of certain physiological conditions (such as
470 experience-dependent song plasticity[92]) or pathological conditions. For example, in

471 the event that the auditory feedback of adult songbirds undergoes modifications or is
472 absent, LMAN-RA pathway will facilitate variations in the signal output of HVC-RA
473 pathway and induce changes in vocalization.[93] It was reported that by activating
474 different nAChR subtypes, long-term synaptic plasticity could be induced in the
475 LMAN-RA pathway of adult male zebra finches through tetanic stimulus,[65]
476 suggesting the potential cholinergic regulation of LMAN-RA pathway in adulthood.
477
478 **Cholinergic modulation of LMAN-RA pathway mediated by mAChRs may be a**
479 **potential mechanism for perception and selection of birdsongs in females**
480 Zebra finches are a species among passerines that exhibits distinct sexual
481 dimorphism in both appearance and song behavior. Adult male birds attract females
482 through singing, whereas females do not sing.[94] Correspondingly, most of song
483 control nuclei in males are much larger than those in females.[95] Studies have
484 demonstrated that the neurons within HVC and RA of females are smaller and less
485 numerous than those of males, so the volumes of the two nuclei in females are
486 considerably smaller than those in males.[96] Such a discrepancy is associated with sex
487 hormones[97] and may even be linked to sex-specific transcriptomes.[8] It has been
488 reported that RAPNs' excitability of adult female zebra finches is significantly lower
489 than that of males.[98] Our previous studies have demonstrated that both excitatory and
490 inhibitory synaptic transmission of RAPNs have sexual dimorphism. In adult female
491 zebra finches, both the frequency and amplitude of RAPNs' spontaneous EPSCs
492 (sEPSCs) and mEPSCs are notably lower than those of males.[99] Moreover, both the
493 frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) and
494 mIPSCs of RAPNs in females are also conspicuously lower than those in males.[84] A
495 study has shown that neuropeptides expression in the neurons of forebrain song
496 control nuclei in female zebra finches is significantly lower than that in males.[100]
497 However, the in-depth research regarding the effect of neurotransmitters on the song
498 related nuclei of female songbirds is rather limited.
499 Previous immunohistochemical results demonstrated that the size and density of
500 ChAT-immunoreactive somata projected from VP to HVC and RA in males are
501 significantly higher than those in females,[101] and the density of ChAT immunoreactive
502 positive-labeled fibers around area X in males is also conspicuously greater than that
503 in females,[47] indicating sex differences in the cholinergic modulation of song control
504 system. Our present study showed that, similar to males, cholinergic signaling can

significantly reduce RAPNs' mEPSCs in adult female zebra finches via mAChRs rather than nAChRs, indicating that RAPNs' excitatory synaptic afferents in adult females is regulated by cholinergic signaling, and the role of mAChRs predominates. To our knowledge, this should be the first time to provide direct evidence for cholinergic modulation of female song control nuclei.

Studies have indicated that the development and formation of HVC-RA song premotor pathway in female zebra finches is incomplete.[102-104] Moreover, it has been reported that sex differences in the myelination of zebra finch HVC-RA tract are significant, and the degree of myelination in females is lower than that in males and remains unchanged with development.[105] Additionally, the quantity of RAPNs in females is markedly less than that in males.[106] These pieces of evidence seemingly offer a rational explanation for the fact that females do not sing. Nevertheless, it has still been proved that female zebra finches have HVC-RA projection fibers like males.[107] Meanwhile, a study of Wang et al. demonstrated that electrical stimulation of HVC-RA projection fibers in adult female zebra finches could result in the corresponding neural activity in RA, indicating the presence of HVC-RA synaptic transmission in females.[108] In accordance with this finding, our present results indicate that RAPNs' eEPSCs can be recorded by electrically stimulating HVC-RA projection fibers in adult female zebra finches, reconfirming the presence of HVC-RA synaptic transmission in adult females. However, we found that, in contrast to males, cholinergic signaling has no effect on HVC-RAPN eEPSCs in females, suggesting that HVC-RAPN excitatory synaptic transmission of females is not regulated by cholinergic signaling. This result indicates that cholinergic modulation of HVC-RA song premotor pathway in adult zebra finches exhibits a typical sex dimorphism.

Among all song nuclei, only the LMAN of females is approximately the same in volume as that of males.[109] Consistent with males, the LMAN of female zebra finches likewise forms neural connections projected to RA in early developmental stage.[103] Moreover, a study has found that in adulthood, the number of synapses in the LMAN of females is significantly higher than that of males.[110] However, it has also been found that LMAN neurons projecting to RA in adult female zebra finches are much less than that in adult males, which might be caused by the massive loss of LMAN-to-RA projection neurons in females during the process of sexual differentiation and song learning.[111] Our further examination revealed that cholinergic signaling significantly reduces the amplitude of RAPNs' eEPSCs evoked by electrically

stimulating the LMAN-RA projection fibers of adult female zebra finches. Moreover, this process is accompanied by the alteration of PPR, indicating that the presynaptic mechanism participated in cholinergic regulation on LMAN-RAPN excitatory synaptic transmission in females. However, unlike the cholinergic regulation of LMAN-RAPN synaptic transmission in males mediated by nAChRs, that in females is mediated by mAChRs rather than nAChRs, which is in line with the receptor mechanism of cholinergic signaling regulating RAPNs' mEPSCs in females. Like zebra finches, in another songbird cowbirds (Molothrus ater), only males sing. Although females do not sing, they can distinguish males by perceiving birdsongs. Research has shown that the volume of female LMAN and the quantity of neurons therein are positively correlated with mating choices, indicating that non-singing females may perceive and choose male songs by LMAN.[112] Hence, female zebra finches, comparable to their male counterparts, might establish memories of song templates (songs heard during juvenile period, such as father's song[113,114]) in the early life stages and gradually develop song perception, among which LMAN may assume a pivotal role.[110] Based on this clue, the cholinergic modulation of LMAN-RA pathway in females might be implicated in the procedure of their perception and selection of male songs.

Truthfully, the perception of auditory events in female zebra finches is not necessarily less competent than that in males.[115-117] Previous research has verified that in female canaries, HVC plays a crucial role in the perception of conspecific song.[118] Further research indicates that the HVC-RA pathway of female zebra finches can mediate predictive call timing in the same way as that of males, and the adaptive timing plasticity of females is stronger than that of males.[115] However, considering the substantial sex dimorphism in the structure and function of HVC-RA pathway, the mechanisms of song perception in females and males may not be identical. Drawing on the interaction and relationship between HVC-RA and LMAN-RA pathways of males, and based on the findings of predecessors and ours, we put forward a mechanism hypothesis that cholinergic signaling impacts the perception and/or selection of male songs in adult female zebra finches by regulating song premotor circuits (Figure 7B). Although cholinergic signaling exerts no influence on HVC-RAPN excitatory synaptic transmission of adult females, it can reduce LMAN-RAPN excitatory synaptic transmission via mAChRs, weaken the dependence on the auditory feedback of LMAN-RA pathway, and thereby relatively strengthen the song

perception (including conspecific songs, and predictive call timing) and/or preference selection of female HVC-RA pathway for males.

**Limitations of the study**

While the roles of different cholinergic receptors were unambiguously identified by pharmacological means, the impact of inhibitory interneuron network within RA on RAPNs could not be precluded in in vivo experiments. The cholinergic modulation of RAPNs' inhibitory synaptic afferents and whether it enhances or attenuates the effect of cholinergic signaling on birdsongs require further investigation. Our hypothesis regarding the cholinergic receptor mechanism in the song learning process of juveniles also remains to be verified, and we are currently attempting to undertake this work. Additionally, the hypothesis concerning the significance of cholinergic modulation on song premotor circuits in females still requires elaborately designed behavioral experiments for validation.

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## AUTHOR CONTRIBUTIONS

Investigation, Ning Xu, Yutao Zhang, and Yalun Sun; methodology, Wei Meng and Songhua Wang; data curation, Ning Xu, Yutao Zhang, Yalun Sun, and Xueqing Song; formal analysis, Wei Meng, Ning Xu, YangYang Cao, and Xinqi Yang; writing – original draft, Wei Meng and Songhua Wang; writing – review & editing, Wei Meng and Ning Xu; conceptualization, Wei Meng; resources, supervision, and project administration, Wei Meng; funding acquisition, Wei Meng and Songhua Wang.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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# STAR★Methods

## Key resources table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Carbachol, CAR | MCE | Cat.# HY-B1208 |
| Atropine sulfate, ATR | MCE | Cat.# HY-B1205 |
| Mecamylamine, MEC | MCE | Cat.# HY-B1395 |
| 1,1-dimethyl-4-phenylpiperazinium iodid, DMPP | MCE | Cat.# HY-W009190 |
| Oxotremorine M iodide, OM | MCE | Cat.# HY-101372A |
| Tetrodotoxin, TTX | Sigma | CAS: 4368-28-9 |
| Picrotoxin, PTX | Sigma | CAS: 124-87-8 |
| Software and algorithms | | |
| Sound Analysis Pro 2011 | Wenchan Zhao et al. ,2019 | soundanalysispro.com |
| Cool Edit Pro 2.0 | Adobe Systems | N/A |
| MiniAnalysis 6.0 | Synaptosoft | N/A |
| Clampfit 10.7 | Molecular Devices | N/A |
| Adobe Illustrator 2021 | | |
| Origin Pro 8.0 | | |
| Graphpad Prism 9.5 | | |
| Other | | |
| 3.5mm Cannula-Single | RWD | Cat.# 62004 |
| 4.0mm Injector-Single | RWD | Cat.# 62204 |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for data should be directed to and will be fulfilled by the lead contact, Wei Meng (meng7883@163.com).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The original data generated during the current study are available from the lead contact upon requests.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this work is available from the lead contact upon requests.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Ethics statement**

All of the work described in this study was approved by the Institutional Animal Care and Use Committee of Jiangxi Science and Technology Normal University (3601020137931).

**Animals**

The adult male and female zebra finches (Taeniopygia guttata) were obtained from a reputable supplier. These birds, all of which were older than 120 days, had previously been raised in a spacious aviary under a light/dark cycle of 14 hours of light and 10 hours of darkness, at a temperature of 24 ℃. Data were collected from a total of 81 adult males and 65 females.

**Slice preparation**

Following deep anesthesia, the brain was carefully extracted. Subsequently, the fresh brain slices were sectioned using a vibrating microtome (7000 smz; Campden Instruments, UK) with a thickness of 250 μm in the coronal plane, and immediately transferred to ice-cold slice solution with an initial pH of 7.3 – 7.4, osmolarity of 330–340 mOsm, bubbled with 95% O2 and 5% CO2 carbogen. The slice solution was composed of 62.5mM NaCl, 5mM KCl, 28 mM NaHCO3, 248 mM sucrose, 1.3mM MgSO4·7H2O, 10mM glucose, and 1.26mM NaH2PO4·H2O. The slices were transferred to a holding chamber containing oxygenated artificial cerebrospinal fluid (ACSF) at 35°C. The ACSF was composed of 125mM NaCl, 2.5mM KCl, 1.2mM MgSO4·7H2O, 1.27mM NaH2PO4·H2O, 25mM NaHCO3, 25mM glucose, and 2.0mM CaCl2, pH 7.3-7.4. The slices were incubated for at least 0.5 h and equilibrated to room temperature prior to electrophysiological recording.

**Patch-clamp electrophysiology**

The visualization of RA in brain slices was achieved using infrared differential interference contrast microscopy (IR-DIC) through a microscope (BX51WIF, Olympus, Tokyo, Japan). Whole-cell patch clamp recordings were conducted at a temperature of approximately 24 °C. Slices were perfused with carbogen-bubbled ACSF at a rate of 1-2 ml/min, while neurons were visualized under a 40× water immersion lens. Patch pipettes were fabricated from standard borosilicate capillary

glass (BF150-117-10, Sutter Instruments, CA, USA) using a Flaming/Brown micropipette puller (P-1000, Sutter Instruments, CA, USA). All recording pipettes had an open-tip resistance ranging from 3.0 to 6.0 MΩ in the bath solution. The patch pipettes were filled with a solution containing the following concentrations: 120 mM KMeSO4, 5 mM NaCl, 10 mM HEPES, 2 mM EGTA, 5 mM QX-314, 2 mM ATP, and 0.3 mM GTP (pH adjusted to range 7.3–7.4; osmolarity set at 340 mOsm). RAPNs exhibit regular spontaneous firing of action potentials and demonstrate slow time-dependent inward rectification of hyperpolarizing current pulses. RA interneurons do not display spontaneously firing action potentials, lack time-dependent inward rectification of hyperpolarizing current pulses, and exhibit irregular spiking in response to depolarizing current injection.[77,119]

To record the miniature excitatory postsynaptic currents (mEPSCs) of RAPNs, a concentration of 1 μM TTX was applied to ASCF in order to block spontaneous events driven by intrinsic Na+ channel-mediated action potentials. During eEPSCs recordings of RAPNs, a bath application of 150 μM picrotoxin (PTX) was employed to block GABAA receptor-mediated inhibitory synaptic currents. Reported holding potentials have been corrected for corresponding liquid junction potential. The signals underwent sampling at 10 kHz and filtering at 2 kHz using the MultiClamp 700B amplifier (Molecular Devices, CA, USA). EPSCs were evoked through a concentric needle electrode placed in the afferent trace of HVC (as shown in Figure 3A, 5A) or LMAN (as shown in Figure 4A, 5A).[120] The paired-pulse ratio (PPR), which quantifies the relative amplitude of the second pulse compared to the first pulse of evoked excitatory postsynaptic currents, was assessed at an inter-pulse interval of 50 ms. Alterations in PPR reflect involvement of presynaptic mechanisms.

**Drug Application**

CAR (30 μM); OM (an agonist of mAChRs, 10 μM); ATR (an antagonist of mAChRs, 10 μM); DMPP (an agonist of nAChRs, 10 μM); MEC (an antagonist of nAChRs, 10μM). The effects of these drugs on RAPNs were assessed through bath perfusion.

**Song recording**

Song recordings were conducted in a recording studio measuring 2.1 × 1.2 × 1 m, equipped with a TAKSTAR directional microphone (Guangdong Victory Electronics

Co. Ltd., Guangzhou, China; frequency range: 50 – 20000 Hz) and a glass window (20 × 40 cm). During the recording sessions, a male bird was placed in a cage near the window within the recording studio and able to see a female bird positioned outside. Each bird's vocalizations were recorded per day between 8:00 am and 11:00 am. Song capturing was performed using Cool Edit Pro 2.0 (sampling rate: 44100 Hz; channels: stereo; resolution: 16-bit).

**In vivo microinjection**

Birds were anesthetized using isoflurane inhalation (1.5%-2%) and placed in a stereotaxic apparatus. Cannulas were implanted into bilateral RA (M/L: ± 2.47; A/P: 0.7; D/V: left 2.8, right 3) (n = 16 birds). After birds recovered from surgery, microjection injectors were inserted into cannulas and connected to a syringe pump (R462, RWD, Guangdong, China) through flexible tubing. Outflow was continually monitored throughout the duration of the experiment. Solutions were exchanged to either phosphate buffered saline (PBS, for control experiments) or 1 mM CAR; 1 mM OM; 1 mM DMPP, after a two to three-hour baseline period (flow rate held constant at 1-1.5 μL/ min; solutions exchanged at the same time each day across experiments).

**Analysis of song features**

We quantified song stereotypy by calculating the similarity to own songs and entropy of the motifs using Sound Analysis Pro 2011. Thirty or Sixty motifs within the song were used to assess the percentage similarity and entropy in Pre and during drug administration.Higher similarity and lower entropy indicate greater stereotypy.[121-123]

**Quantification and statistical analysis**

The data were acquired using Clampfit 10.7 through a Digidata 1550B (Molecular Devices, CA, USA) with 10 kHz sampling frequency. Analysis of mEPSCs was performed using Clampfit 10.7, Mini 6 and Origin Pro 8.0 (OriginLab, MA, USA). The average values of inter-event interval (IEI) and amplitude for events in the control and drug-administered groups were compared. Paired t-tests were conducted to statistically compare the mean averaged values for each group, unless otherwise specified. The significance levels of IEI and amplitude shifts were determined by employing the nonparametric Kolmogorov-Smirnoff (K-S) test to assess cumulative

probability distributions. The data are presented as mean ± SEM. The software tools employed for the creation and analysis of experimental diagrams were Adobe Illustrator 2021(Adobe, CA, USA), Origin Pro 8.0, and Graphpad Prism 9.5(GraphPad Software, CA, USA).

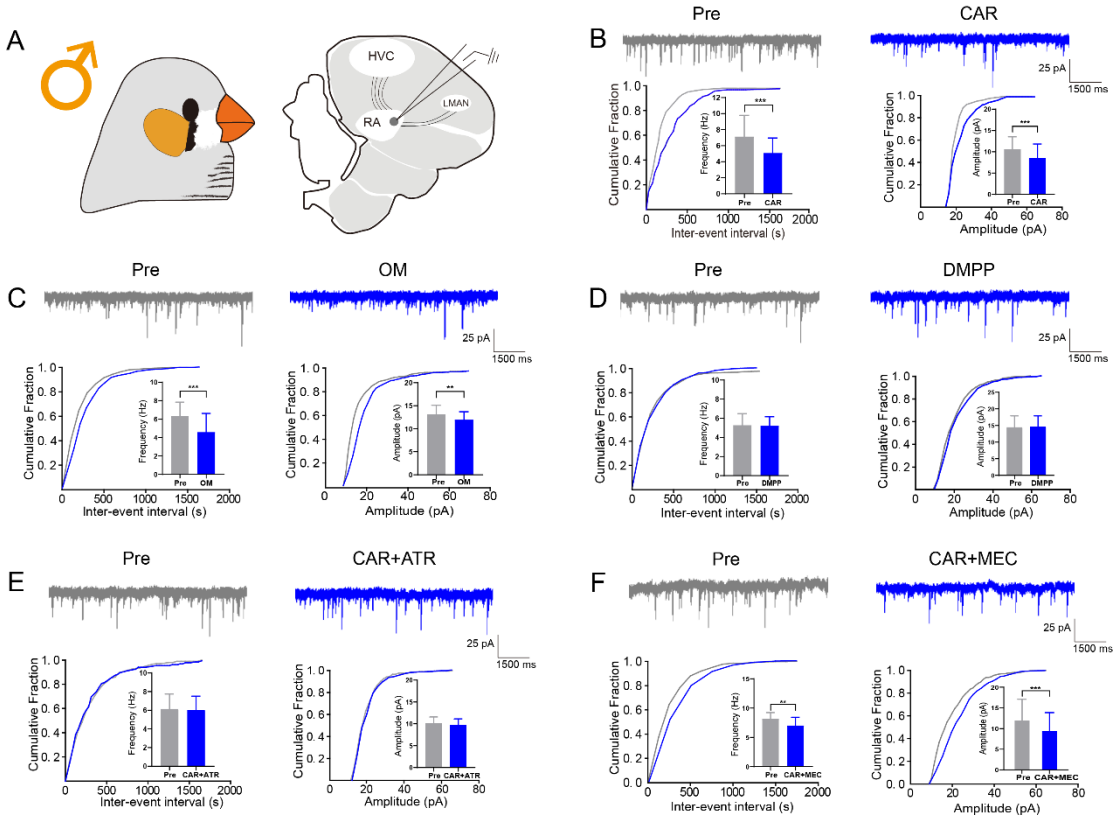


**Figure 1 Cholinergic modulation of RAPNs' mEPSCs in adult male zebra finches**

(A) Experiment schematic. In vitro whole-cell patch clamp recording of RAPNs' mEPSCs in adult male zebra finches.

(B) Top, example traces of adult male RAPNs' mEPSCs in Pre (gray) and CAR (blue); Bottom, quantification of mEPSCs' frequency (left) and amplitude (right) in Pre and CAR, n = 9.

(C-F) Similar to (B). OM (C), n = 8; DMPP (D), n = 9; CAR+ATR (E), n = 7; CAR+MEC (F), n = 8. ** $p \leq 0.01$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

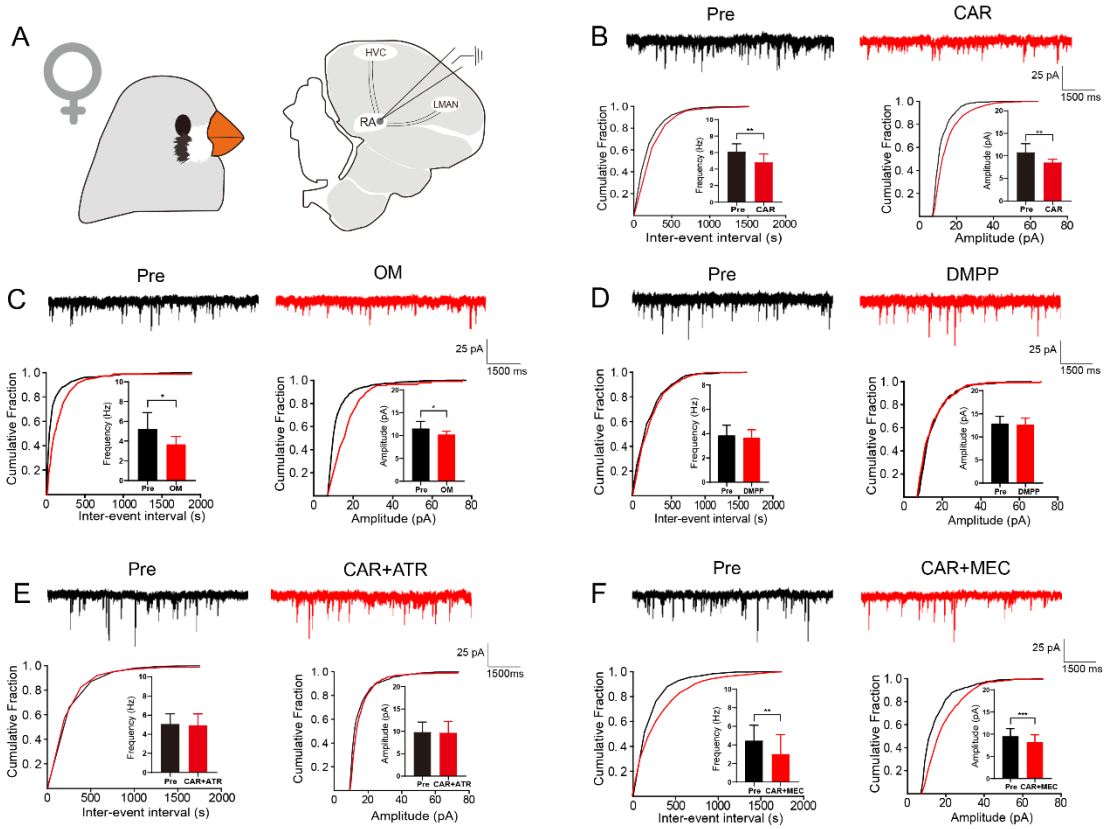


**Figure 2 Cholinergic modulation of RAPNs' mEPSCs in adult female zebra finches**

(A) Experiment schematic. In vitro whole-cell patch clamp recording of RAPNs' mEPSCs in adult female zebra finches.

(B) Top, example traces of adult female RAPNs' mEPSCs in Pre (black) and CAR (red); Bottom, quantification of mEPSCs' frequency (left) and amplitude (right) in Pre and CAR, n = 9.

(C-F) Similar to (B). OM (C), n = 8; DMPP (D), n = 9; CAR+ATR (E), n = 9; CAR+MEC (F), n = 8. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

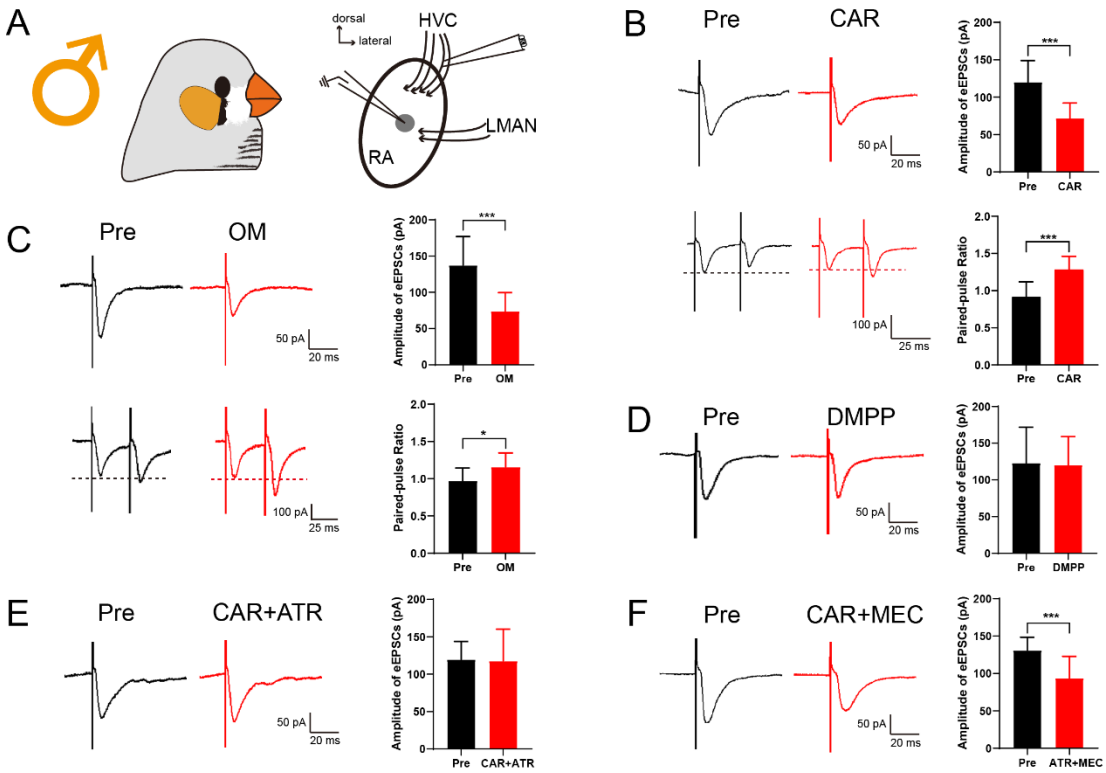


**Figure 3 Cholinergic modulation of HVC-RAPN eEPSCs in adult male zebra finches**

(A) Experiment schematic. In vitro whole-cell patch clamp recording of HVC-RAPN eEPSCs in adult male zebra finches.

(B) Top, example traces of adult male HVC-RAPN eEPSCs in Pre (black) and CAR (red). Quantification of eEPSCs' amplitude in Pre and CAR, n = 10; Bottom, example traces of eEPSCs in paired pulse stimulation. Quantification of paired-pulse ratio in Pre and CAR, n = 7.

(C) Similar to (B). OM: Top, n = 8; Bottom, n = 7.

(D-F) Example traces of adult male HVC-RAPN eEPSCs in Pre (black), DMPP (D, red), CAR+ATR (E, red) and CAR+MEC (F, red). Quantification of eEPSCs' amplitude in Pre, DMPP (D, n = 11), CAR+ATR (E, n = 8), and CAR+MEC (F, n = 11). * $p \leq 0.05$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

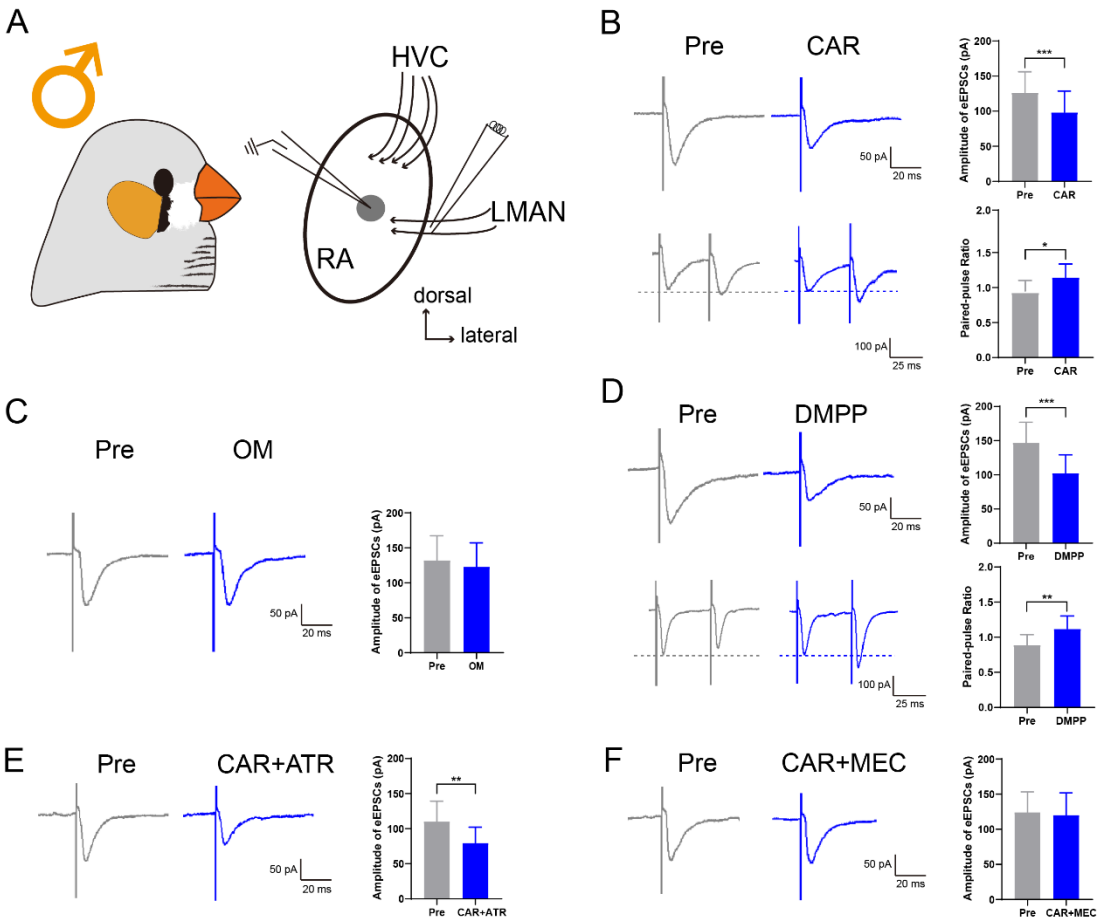


**Figure 4 Cholinergic modulation of LMAN-RAPN eEPSCs in adult male zebra finches**

(A) Experiment schematic. In vitro whole-cell patch clamp recording of LMAN-RAPN eEPSCs in adult male zebra finches.

(B) Top, example traces of adult male LMAN-RAPN eEPSCs in Pre (gray) and CAR (blue). Quantification of eEPSCs' amplitude in Pre and CAR, n = 9; Bottom, example traces of eEPSCs in paired pulse stimulation. Quantification of paired-pulse ratio in Pre and CAR, n = 8.

(C) Example traces of adult male LMAN-RAPN eEPSCs in Pre (gray) and OM (blue). Quantification of eEPSCs' amplitude in Pre and OM, n = 8.

(D) Similar to (B). DMPP: Top, n = 8; Bottom, n = 8.

(E-F) Similar to (C). CAR+ATR (E), n = 10; CAR+MEC (F), n = 8. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

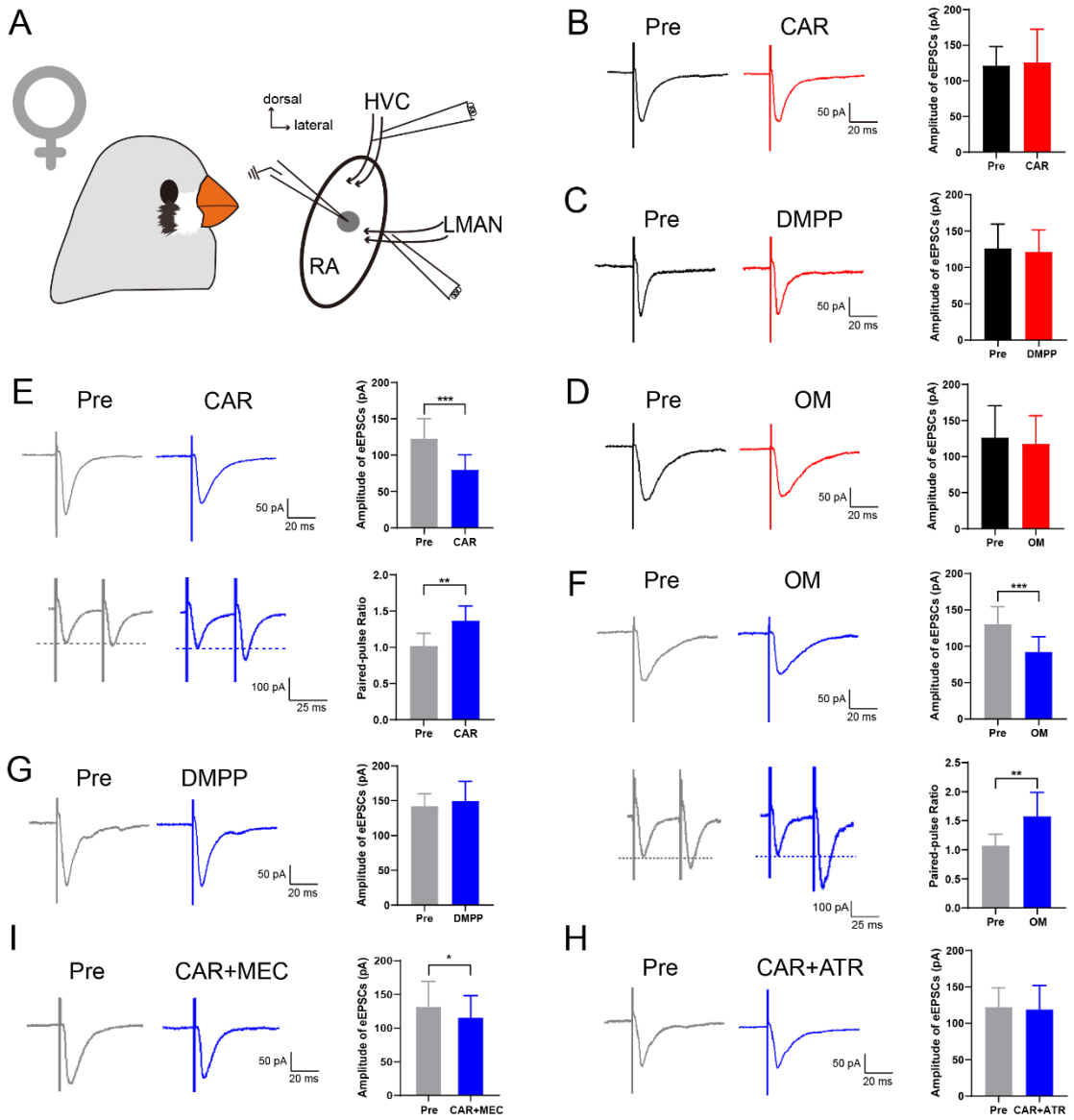


**Figure 5 Cholinergic modulation of HVC-RAPN and LMAN-RAPN eEPSCs in adult female zebra finches**

(A) Experiment schematic. In vitro whole-cell patch clamp recording of HVC-RAPN and LMAN-RAPN eEPSCs in adult female zebra finches.

(B) Example traces of adult female HVC-RAPN eEPSCs in Pre (black) and CAR (red). Quantification of eEPSCs' amplitude in Pre and CAR, n = 10.

(C-D) Similar to (B). DMPP (C), n = 8; OM (D), n = 9.

(E) Top, example traces of adult female LMAN-RAPN eEPSCs in Pre (gray) and CAR (blue). Quantification of eEPSCs' amplitude in Pre and CAR, n = 10; Bottom, example traces of eEPSCs in paired pulse stimulation. Quantification of paired-pulse ratio in Pre and CAR, n = 7.

(F) Similar to (E). OM: Top, n = 8; Bottom, n = 7.

(G) Example traces of adult female LMAN-RAPN eEPSCs in Pre (gray) and DMPP (blue). Quantification of eEPSCs' amplitude in Pre and DMPP, n = 8.

(H-I) Similar to (G). CAR+ATR (H), n = 10; CAR+MEC (I), n = 9. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

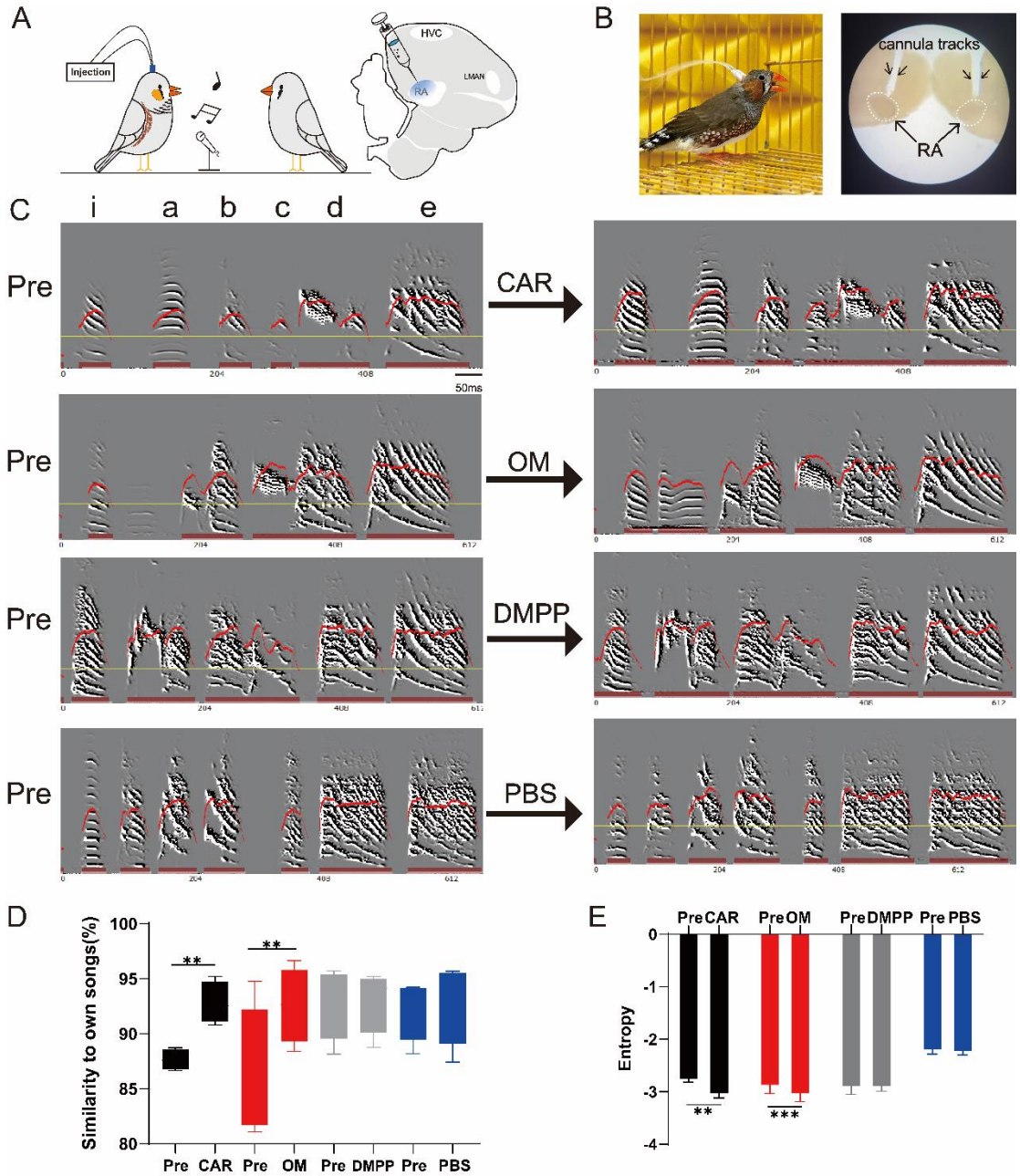


**Figure 6 Cholinergic signaling within RA enhances song stability of adult male zebra finches via mAChRs rather than nAChRs**

(A) Experiment schematic. In vivo pharmacological microinjection onto bilaterally RA and song recording.

(B) Left, a free-moving adult male zebra finch in a recording studio with cannulas bilaterally implanted over RA; Right, cannula tracks in a coronal section.

(C) Example spectrograms in Pre and CAR, OM, DMPP or PBS. 'iabcde' indicates the syllables constituting a song motif.

(D) Similarity to own songs in Pre and CAR (black, n = 4), Pre and OM (red, n = 4), Pre and DMPP (gray, n = 4), Pre and PBS (blue, n = 4).

(E) Song entropy in Pre and CAR (black, n = 4), Pre and OM (red, n = 4), Pre and DMPP (gray, n = 4), Pre and PBS (blue, n = 4). ** $p \leq 0.01$, *** $p \leq 0.001$, paired *t*-test. Plots represent mean ± SEM.

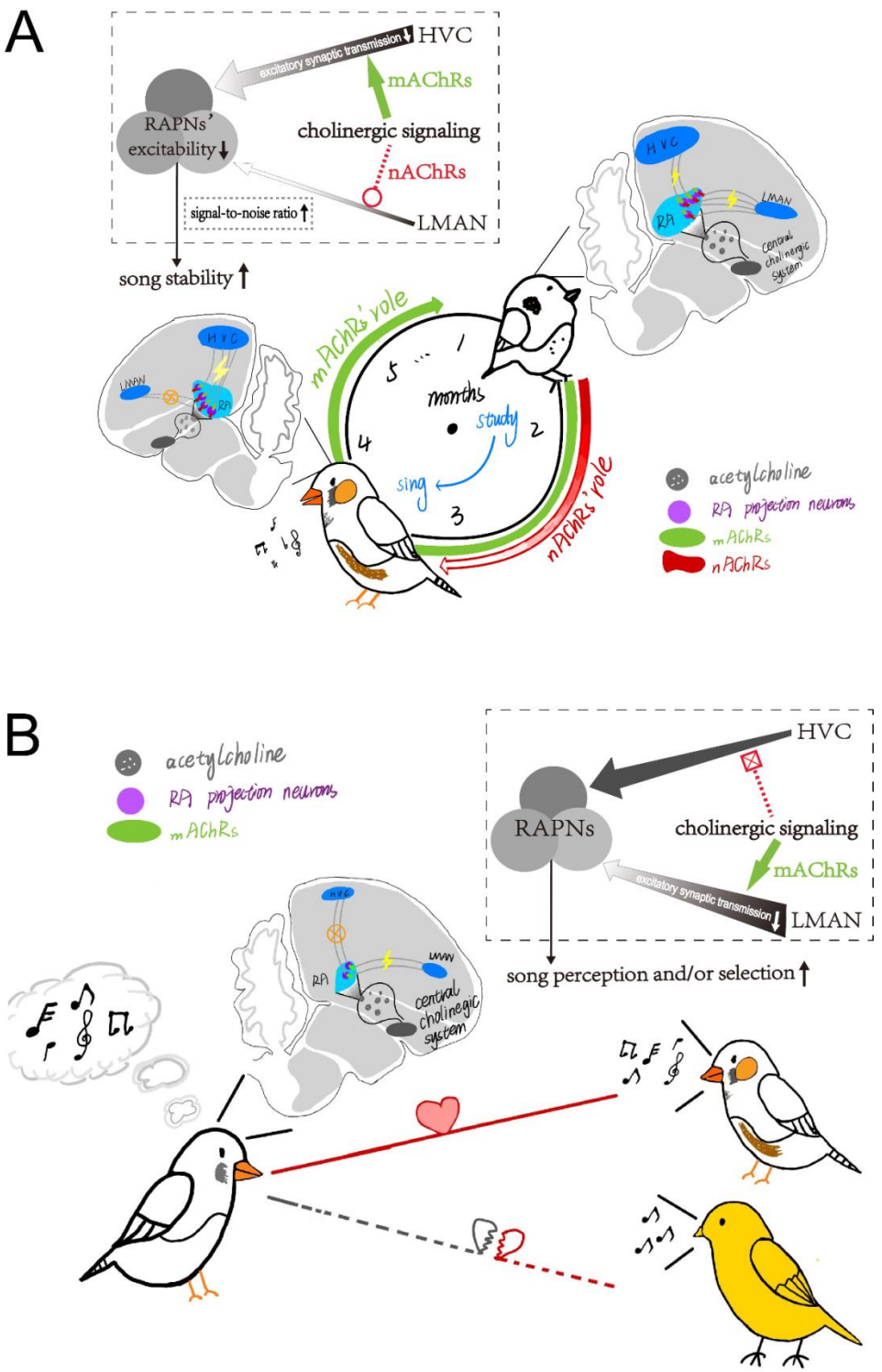


**Figure 7 Mechanism hypotheses on the cholinergic modulation of song premotor circuits in male and female zebra finches**

(A) A hypothesis regarding the mechanism through which cholinergic signaling influences song production by regulating song premotor circuits in male zebra finches.

(B) A hypothesis regarding the mechanism through which cholinergic signaling impacts the perception and/or selection of male songs by regulating song premotor circuits in adult female zebra finches. Details in "DISCUSSION".

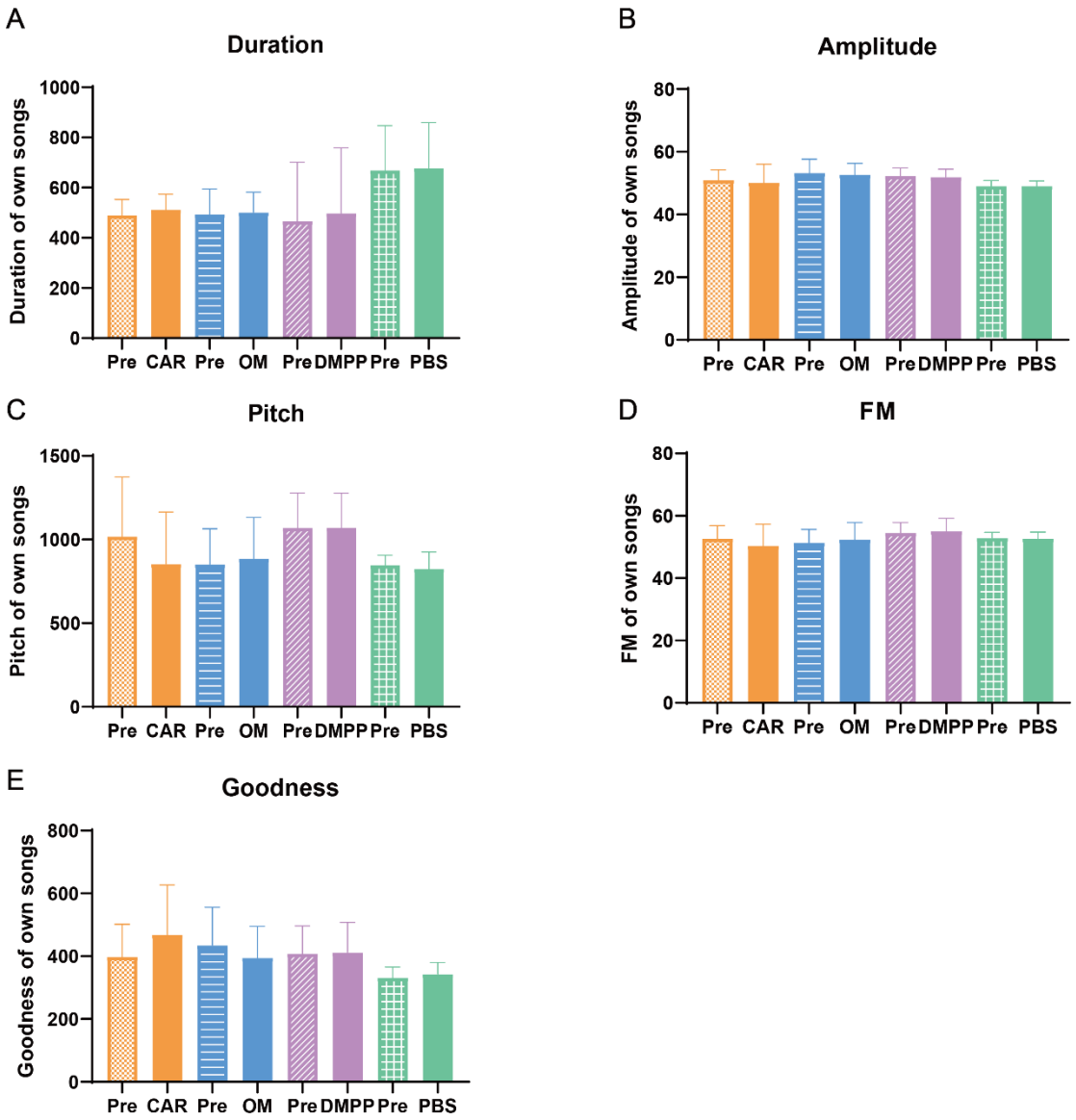


**Figure S1 Other song acoustic characteristics are not influenced by cholinergic signaling within RA**

(A) Duration of own songs in Pre and CAR (orange, n = 4), Pre and OM (blue, n = 4), Pre and DMPP (purple, n = 4), Pre and PBS (green, n = 4).

(B) Amplitude of own songs in Pre and CAR (orange, n = 4), Pre and OM (blue, n = 4), Pre and DMPP (purple, n = 4), Pre and PBS (green, n = 4).

(C) Pitch of own songs in Pre and CAR (orange, n = 4), Pre and OM (blue, n =4), Pre and DMPP (purple, n = 4), Pre and PBS (green, n = 4).

(D) FM of own songs in Pre and CAR (orange, n = 4), Pre and OM (blue, n = 4), Pre and DMPP (purple, n = 4), Pre and PBS (green, n = 4).

(E) Goodness of own songs in Pre and CAR (orange, n = 4), Pre and OM (blue, n = 4), Pre and DMPP (purple, n = 4), Pre and PBS (green, n = 4). Paired *t*-test. Plots represent mean ± SEM.