Resurgent Na⁺ currents promote ultrafast spiking in projection neurons that drive fine motor control

Benjamin M. Zemel¹, Alexander A. Nevue², Andre Dagostin¹, Peter V. Lovell², Claudio V. Mello², Henrique von Gersdorff^{1,3}

¹Vollum Institute, Oregon Health and Science University, Portland, Oregon 97239

²Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, Oregon 97239

³Oregon Hearing Research Center, Oregon Health and Science University, Portland, Oregon 97239

Corresponding Authors:

Henrique von Gersdorff (vongersd at ohsu.edu) and Claudio V. Mello (melloc at ohsu.edu)

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The underlying mechanisms that promote precise spiking in upper motor neurons controlling fine motor skills are not well understood. Here we report that projection neurons in the adult zebra finch song nucleus RA display: 1) robust high-frequency firing, 2) ultra-short half-width spike waveforms, 3) superfast Na⁺ current inactivation kinetics and 4) large resurgent Na⁺ currents (I_{NaR}). These spiking properties closely resemble those of specialized pyramidal neurons in mammalian motor cortex and are well suited for precise temporal coding. They emerge during the critical period for vocal learning in males but not females, coinciding with a complete switch of modulatory Na⁺ channel subunit expression from Navβ3 to Navβ4. Dynamic clamping and dialysis of Navβ4's C-terminal peptide into juvenile RA neurons provide evidence that this subunit, and its associated I_{NaR} , promote neuronal excitability. We propose that Navβ4 underpins I_{NaR} that facilitates precise, prolonged, and reliable high-frequency firing in upper motor neurons.

Introduction

The speed and accuracy of muscle control and coordination depends on the spiking activity of upper motor neurons¹. In mammals, a subclass of layer 5 pyramidal neurons (L5PNs) project from the motor cortex to various targets in the brainstem and spinal cord. These cells are involved in specific aspects of fine motor control and they produce narrow half-width action potentials (APs)^{2,3}. Changes to their intrinsic properties have been implicated in facilitating the learning of complex motor skills in some species⁴⁻⁸. Notably, primates and cats possess varying numbers of very large L5PNs with wide-caliber myelinated axons, fast AP conduction velocities and ultra-narrow AP spikes⁹⁻¹¹. These specialized Betz-type cells, first discovered in primates, send projections that often terminate directly onto lower motor neurons⁷, and are thought to be involved in highly refined aspects of motor control^{1,5,9,10}.

Birds display a diverse array of complex behaviors and cognitive skills ranging from elaborate nest building and tool usage to episodic memory and vocal mimicry¹²⁻¹⁴. Remarkably, birds accomplish this without a typical six-layered neocortex, which underpins the capacity for complex motor skills in mammals^{15,16}. Nonetheless, avian pallial nuclei form microcircuits that appear

analogous to those in the mammalian neocortex¹⁵⁻¹⁹. In songbirds, the robust nucleus of the arcopallium (RA) plays key roles in singing and provides direct descending projections to brainstem motor neurons that innervate the avian vocal organ (syrinx; Fig. 1a)²⁰⁻²² and respiratory muscles²³. RA projection neurons (RAPNs) can thus be considered analogous to L5PNs in motor cortex¹⁶⁻¹⁹. Indeed, these cells share important features, like wide-caliber myelinated axons and multiple spine-studded basal dendrites²⁴, although RAPNs lack the large, multilayer-spanning apical tufted dendrites that are a hallmark of L5PNs²⁵⁻²⁷. However, detailed knowledge of the ion channel composition, biophysical properties and firing patterns of RAPNs is limited. Therefore, it is still unclear to what extent they function in an analogous manner to L5PNs.

Zebra finch RAPNs face considerable spiking demands during singing, which requires superfast, temporally precise coordination of syringeal and respiratory musculature²⁸⁻³⁰. As the adult male sings, RAPNs exhibit remarkably precise spike timing (variance ~0.23 ms)³¹. RAPNs also exhibit increased burstiness during the developmental song learning period, their instantaneous firing rates changing from 100-200 Hz when they produce immature vocalizations (subsong) to 300-600 Hz when song becomes mature (crystallized). Average overall spike rates of RAPNs increase from 36 Hz at subsong to 71 Hz in adults³². Song maturation thus correlates with reduced variability in the timing of increasingly high frequency bursts with a refinement of single spike firing precision. Importantly, nerve firing rates of >75 Hz are required for force summation in the superfast syrinx muscles³³. This high spike frequency in RAPNs during song production is energetically demanding as indicated by increased staining for cytochrome C in maturing males, but not female zebra finches³⁴.

Songbird RAPNs thus seem to share with Betz-type L5PNs similar evolutionary pressure for fast and precise signaling, a constraint that can lead to neurons in unrelated species sharing similar expression patterns for a specific repertoire of ion channels. A good example are electric fish species from different continents, which have independently evolved electrocytes exhibiting similar physiology and molecular convergence^{35,36}. Indeed, high-frequency firing electrocytes with extremely narrow AP spikes co-express fast activating Na⁺ and K⁺ channels^{37,38}.

Here we explored the excitability of zebra finch RAPNs, posing the following question: Are the properties of Na⁺ currents in RAPNs specialized to produce narrow, non-adapting spikes that enable precise high-frequency burst firing? We demonstrate that RAPNs share strikingly similar spiking properties and ion channel subunits with Betz-type L5PNs. We provide strong evidence linking the presence of voltage-gated Na⁺ channel auxiliary $\beta 4$ subunit (Nav $\beta 4$) to large resurgent Na⁺ currents (I_{NaR}) in RAPNs. We also provide compelling evidence that the ability of maturing RAPNs to produce narrow APs strongly correlates with increasing Nav $\beta 4$ mRNA expression during the critical period for vocal learning in males, but not females that do not sing. We conclude that Nav $\beta 4$ and related I_{NaR} likely play an important role in fine tuning the intrinsic excitability of RAPNs. We propose these biophysical properties allow a subclass of large upper motor neurons to operate as highly specialized control units for the production of rapid and precise movements.

Results

Spontaneous and evoked spiking in RAPNs

Whole-cell current clamp recordings performed in adult male zebra finch brain slices revealed that RAPNs fire spontaneously, with tonic activity in the absence of synaptic input. Action potential (APs) spikes had large amplitudes and narrow, submillisecond durations with a prominent after-depolarization that facilitated the production of subsequent spikes (Fig. 1b-d; spikes at ~24°C; see Table 1). Recorded RAPNs were easily distinguished from local inhibitory GABAergic

interneurons based on differences in their spiking properties and morphology (Supplementary Fig. 1). These results are consistent with previous studies^{24,39-42}. However, phase plane plots from the first derivative of these spontaneous APs revealed a biphasic AP upstroke, (Fig. 1e), which has not been previously described in songbirds. This biphasic AP is a hallmark of L5PNs in mammalian neocortex and suggests a high density of voltage-gated Na⁺ channels (Nav) at the axon initial segment⁴³. The phase plots also revealed large maximum rates of depolarization and repolarization (Fig. 1e; Table 1), which likely facilitates high frequency firing during *in vivo* song production³².

At physiological avian body temperature (~40°C⁴⁴) the spontaneous firing frequency increased four-fold compared to room temperature (Fig. 1b and f; Table 1;⁴⁵). Importantly, the AP waveform had a smaller amplitude at 40°C and an extremely narrow half-width (~0.2 ms; Fig. 1g; Table 1). The APs also exhibited faster maximum rates of depolarization and repolarization in the phase plot at 40°C (Fig. 1h; Table 1). During positive current injections adult RAPNs produced high-frequency AP trains at 40°C (Fig. 1f). Large amplitude spikes were triggered for the duration of the current injection with only a modest initial adaptation of spike amplitude. In fact, RAPNs showed little change in the maximum rate of depolarization between the 1st and 2nd spikes, despite a faster instantaneous firing frequency (IFF) at 40°C (Fig. 1g; for ~24°C data see Supplementary Fig. 2). We also note that during negative current injections at room temperature, we observed a voltage sag that is consistent with HCN-mediated currents (5.0 ± 0.68 mV between the peak and steady-state voltage during a -150 pA step stimulus), as previously described for RAPNs⁴⁰.

These results show that RAPNs have similar electrophysiological properties to cortical L5PNs that also display spontaneous APs with sub-millisecond half-widths, temperature-sensitive amplitudes, and modestly adapting AP firing during positive current injections^{43,46-48}. Importantly, the prolonged high frequency spiking of RAPNs suggests a high degree of availability of Nav channels. We therefore hypothesized that adult RAPNs contain a large pool of Nav channels and specialized mechanisms that limit their dwell time in non-conducting inactivated states.

Navβ4 expression predicts a robust resurgent Na⁺ current (I_{NaR})

Recently, we have identified several transcripts for voltage-gated Na⁺ channel beta (Navβ) auxiliary subunits as either positive (Navβ4) or negative (Navβ3) markers of adult male RA in the arcopallium⁴⁹. While the functional role of these different Navβ subunits in RA neuronal excitability is unknown, Navβ4 is of particular interest because it promotes narrow spike waveforms and high frequency firing via a resurgent sodium current (I_{NaR})⁵⁰⁻⁵². We therefore hypothesized that Navβ4 might be a key determinant of excitability properties in RA. High expression of Navβ4 mRNA is restricted to RA in the arcopallium of adult male zebra finches⁴⁹, contrasting sharply with Navβ3, which is all but absent (Fig. 2a-b). Navβ4 is thought to promote high frequency firing by limiting classical inactivation of Nav channels and promoting I_{NaR}⁵³. Based on its expression levels, we predicted that neurons within RA would have a larger I_{NaR} than those in the arcopallium outside RA. We thus performed whole-cell voltage clamp recordings in RA sagittal slices from adult male finches (Fig. 2c). Voltage steps from -90 mV to +30 mV elicited large transient sodium currents (I_{NaT}) and, as predicted, subsequent steps to a range of test potentials (+15 mV to -75 mV) yielded robust I_{NaR} (Fig. 2c) with a peak of -3.0 nA ± 0.28 (Mean ± SEM) at the -45 mV test potential (Fig. 2e). The decay kinetics of I_{NaR} was voltage dependent, with single exponential decay time constants ranging from 2 to 45 ms (Supplementary Fig. 3a-b). These results reveal an exceptionally large I_{NaR} in RAPNs compared to those recorded in mammalian⁵² and chicken brainstem neurons⁵⁴.

In sharp contrast to RAPNs, neurons recorded in a caudal arcopallial region outside RA (Fig. 2a; shaded area), that has low Navβ4 expression, showed a much smaller I_{NaR} (Fig. 2d-e). Notably,

the recorded cells outside RA were morphologically distinct from neurons within RA, with relatively smaller somata and dendrites with numerous, thin spines (Supplementary Fig. 1f). Because I_{NaR} is a function of previously opened Nav channels⁵², the expression of which can vary across cells, we normalized peak I_{NaR} measurements to the peak I_{NaT} (ratio of I_{NaR}/I_{NaT}). The average normalized peak I_{NaR} was still much larger in RAPNs compared to neurons outside RA over a range of test potentials (-60 mV to -30 mV), with maximum normalized values of 0.28 ± 0.01 and 0.08 ± 0.01 at -45 mV for RAPNs and neurons outside RA, respectively (Fig. 2f). Decay kinetics also showed differences, with RAPNs exhibiting significantly smaller time constants at depolarized test potentials (-15 to +15 mV; Supplementary Fig. 3b).

Because Nav β 4 has been linked to facilitation of high frequency firing in mammals and chicken^{52,54}, we predicted that RAPNs would be more excitable than neurons outside RA. Indeed, recordings of spontaneous APs proved consistent with this prediction with RAPNs exhibiting narrower APs, larger AP amplitudes and larger maximum rates of depolarization and repolarization than neurons outside RA (Table 1). Furthermore, while neurons both inside and outside RA were capable of increased spiking in response to current injections (Fig. 2g-i), RAPNs produced more spikes per second with a higher instantaneous firing frequency (IFF) for the first two APs at all positive current injections above 200 pA (Fig. 2i-j). These findings point to a strong correlation between Nav β 4 mRNA expression, larger I_{NaR} , and greater intrinsic excitability, suggesting a role for Nav β 4 in regulating intrinsic excitable properties of RAPNs in zebra finches.

Nav β 4 and I_{NaR} increase in parallel in RA during vocal development

Male juvenile zebra finches progress through a critical period of vocal learning, during which vocal practice guided by auditory feedback is required to accurately produce a copy of the tutor song^{12,55}. Zebra finches do not sing prior to ~28 days post hatch (dph). During the next phase (~28 to 45 dph) they produce unstructured vocalizations, referred to as subsong. They next enter a plastic phase (~45 to 90 dph), during which songs become more structured as the tutor refines syllable structure and sequencing guided by auditory feedback. By ~90 dph the song becomes crystallized and highly stereotyped. These developmental changes in song coincide with the growth and maturation of vocal control nuclei in males. In contrast, female finches do not develop song and their song nuclei experience marked reductions in volume, as well as sharp decreases in neuronal cell number and size^{56,57}. The morphology, connectivity and electrophysiological properties of RAPNs also change markedly during the song learning period^{32,41,42,56-61}.

To investigate whether developmental changes in RA are also associated with changes in the expression of Nav β subunits, we performed *in situ* hybridizations for Nav β 4 and Nav β 3 in sagittal brain sections from male zebra finches at ages known to be within the pre-song (20 dph), subsong (35 dph), plastic song (50 dph) and crystallized song (>90 dph) stages of vocal development^{55,62}. We observed an age-dependent increase in Nav β 4 expression level in RA, with significant differences between 20 dph and 35 dph juveniles compared to adults (Fig. 3a-d, and I, black). We also detected an age-dependent increase in the proportion of Nav β 4-expressing cells, progressing from 2% of cells relative to Nissl in pre-song juveniles (20 dph) to 24%, 31%, and 63% in 35 dph, 50 dph, and adult birds respectively, with a significant difference between adult and 20 dph finches (Fig. 3j, black). In contrast, no significant changes in Nav β 4 mRNA expression, or the proportion of positive cells, were detected outside RA across ages (Fig. 3a-d, and i-j, grey). In stark contrast to Nav β 4, we find that Nav β 3 expression level and the proportion of Nav β 3-expressing cells in RA decreased markedly across ages (Fig. 3e-h, and k-l, black), with little evidence of change outside RA (Fig. 3e-h, and k-l, grey). Finally, we note that while Nav β 2 is non-differentially expressed across the arcopallium, Nav β 1 expression is higher in RA of adult males compared to the surrounding arcopallium⁴⁹. We therefore asked whether Nav β 1 is developmentally regulated. Similar to Nav β 4, we find that Nav β 1 expression was not present in

nucleus RA in 20 dph males, contrasting markedly with the strong Navβ1 expression in adults (Supplementary Fig. 4).

Given the changes in Navβ4 expression, we predicted a corresponding age-dependent increase of I_{NaR} in RAPNs. RA could be readily identified in sagittal brain obtained from 20-50 dph male finches via infra-red differential interference microscopy (IR-DIC; Supplementary Fig. 5). Indeed, I_{NaR} was small or absent in RAPNs from 20 dph finches, but its magnitude increased sharply with age (Fig. 4a-b), with significant age-dependent effects observed for both peak I_{NaR} (Fig. 4c) and normalized peak I_{NaR}/I_{NaT} ratios (Fig. 4d). At the -30 mV test pulse, significant differences were found for post-hoc pairwise comparisons of raw and normalized I_{NaR} across all age groups. Importantly, the average normalized peak I_{NaR}/I_{NaT} values were significantly correlated with both the average Navβ4 expression level (Fig. 4e) and the average proportion of Navβ4-expressing cells within RA (Fig. 4f). Therefore, Navβ4 mRNA expression strongly correlates with I_{NaR} in RA, and predicts the increase of I_{NaR} seen in RA across different ages within the vocal learning period.

Age-dependent changes in the activation and inactivation kinetics of I_{NaT}

To address possible concerns that age-dependent differences in our voltage clamp recordings might be affected by space and/or voltage clamp issues, we also performed recordings in frontal slices that transect RA axons near the soma (Fig. 5a-c). For those recordings, we lowered extracellular Na^+ from 119 to 30 mM to decrease the magnitude of I_{Na} and compensated the series resistance electronically from 5 to 1 MΩ. These changes will improve space and voltage clamp. Consistent with sagittal recordings with normal extracellular Na^+ (Fig. 4a-d), we observed a significant age-dependent increase in I_{NaR} and confirmed that the normalized peak I_{NaR} was significantly larger in the RAPNs of adults compared to 20 dph finches, where I_{NaR} was almost undetectable (-30 mV test pulse; Fig. 5d-e). We note that we are likely underestimating I_{NaR} in these experiments as lowering external Na^+ ions disproportionately reduces I_{NaR} ⁵². Importantly, the resulting I-V plots of the peak I_{NaT} from adults and 20 dph finches suggest that these currents are well voltage-clamped under these conditions (Fig. 5f)⁶³. Adults had a larger I_{NaT} amplitude that appeared to activate at more hyperpolarized potentials than that of 20 dph juveniles (Fig. 5f). This likely leads to the faster AP upstroke and decreased threshold in adult RAPNs compared to juveniles (Table 1). In order to quantify the voltage dependence of I_{NaT} we fit the average I-V plots with the following Boltzmann function:

$$I = g_{max}(V - V_{rev})/(1 + \exp((V_{1/2} - V)/k))$$

where g_{max} is the maximum conductance, V_{rev} is the reversal potential, $V_{1/2}$ is the half-maximal activation and k is the slope⁶⁴. Fitting this function to the I-V curves revealed that adults had a notably larger maximal conductance and leftward shifted half-maximal activation compared to juveniles. The values from the fits were as follows for adults and juveniles, respectively: $g_{max} = 174.0$ and 95.0 nS; $V_{rev} = 66.5$ and 60.0 mV, $V_{1/2} = -44.6$ and -30.7 mV, and $k = 6.6$ and 7.3 mV. As predicted by the RAPN firing properties, the averaged I_{NaT} also had a faster onset from the voltage step in adults (0.11 ms) compared to 20 dph juveniles (0.17 ms) (Fig. 5g). In order to quantify the kinetics of I_{NaT} we fit the onset of this current with the following function:

$$I_{NaT} = I_{max}(1 - e^{-t/\tau_a})^N$$

where τ_a is the activation time constant and N is the power law exponent for the apparent number of transitions of I_{NaT} activation as defined by a Markov chain model for a classic Hodgkin-Huxley Na^+ current activation m gate⁶⁵. Interestingly, fitting this function from the onset of I_{NaT} to ~40% of the peak during a +30 mV test pulse yielded an apparent number of activation gates in the

averaged adult I_{NaT} ($N = 10.7$) that greatly deviated from the Hodgkin-Huxley gating scheme ($N = 3$); this deviation was less apparent in the juvenile I_{NaT} ($N = 3.7$; Fig. 5g). Similar fits to I_{NaT} yielded an $N = 5$ at +30 mV steps from nucleated patches of L5PNs⁶⁵ and a $N = 9$ at +10 mV steps in cerebellar granule cells⁶⁶. The time constants of activation were also smaller in adults ($\tau_a = 0.10$ ms) compared to juveniles ($\tau_a = 0.15$ ms) (Fig. 5g). Furthermore, the time constant of inactivation for I_{NaT} was ~ 2-fold smaller in RAPNs from adults (0.33 ± 0.05 ms; mean $\pm$ SE) compared to 20 dph juveniles (0.58 ± 0.04 ms) (Fig. 5h-i). This suggests the presence of a superfast Nav channel inactivation process that precedes the I_{NaR} . This fast inactivation may be mediated in part by the fast block provided by Nav β 4, as suggested in other systems^{51,67}. During these studies we also observed a small persistent Na^+ current in adult RA (0.3 ± 0.1 nA at 100 ms after a voltage step from -90 to -20 mV at steady state; Fig. 5h).

Age-dependent increases in the intrinsic excitability of male finch RAPNs

We next investigated the intrinsic excitability of RAPNs during development using whole-cell current clamp recordings. RAPNs showed spontaneous firing at all ages examined (Table 1; representative spontaneous spikes shown in Fig. 6a). The recorded APs exhibited significant age-dependent decreases in threshold and increases in maximum depolarization and repolarization rates (Fig. 6b-c; Table 1). We also found age-dependent changes in the passive properties, with significant decreases in both the input resistance (R_{in}), consistent with previous findings in male finches⁶¹, and in membrane time constant (Table 1). Although the calculated membrane capacitance (C_m) showed an upward trajectory across ages, we observed a trend toward a decrease from P50 to adults (Fig. 4b; Table 1). This observation mirrors those from a previous study⁴¹. This decrease in C_m may be due to an increase in axonal compact myelination between these ages (Fig. 5b and Supplementary Fig. 5;⁶⁸). Furthermore, by multiplying the average C_m (114 pF) by the maximum dV/dt we calculated a large peak Na^+ current of 61 nA in adult RAPNs produced during a spontaneous AP (Table 1). By comparison, neurons of similar size ($C_m = 80$ pF) within deep cerebellar nuclei of mammals have only 21 nA currents⁶⁹. These estimated peak Na^+ current values increased markedly across age in finches (Table 1), a finding that is supported by voltage clamp experiments (Fig. 5d&f).

RAPNs at all ages examined were capable of increased AP firing in response to positive current injections (Fig. 6d-g, left). However, we observed a significant age-dependent increase in the number of spikes per second produced during current injections. APs in 20 dph finches began failing at current injections > 200 pA. Significant differences were seen when comparing 35 dph juveniles to either 50 dph or adults at current injections ≥ 400 pA (Fig. 6h). We also observed a significant age-dependent increase in the IFF, with significant differences when comparing 20 or 35 dph juveniles to either 50 dph or adults at multiple levels of injected current (Fig. 6i). We thus conclude that both spike number and IFF reached a maximum at 50 dph that persisted through adulthood.

We next examined how features of AP waveforms changed during development. By overlaying the 1st, 2nd and 16th-21st APs elicited by a +500 pA current injection, we were able to observe the effect of repetitive firing on spike amplitude and duration. This analysis revealed a progressive AP broadening and reduction in amplitude in 20 dph birds that began after the 1st AP, eventually reaching a steady state as the train progressed (Fig. 6d-g, middle). These effects were largely attenuated in older juveniles and adults (Fig. 6d-g, middle). Consistent with this finding, phase plane plots of 20 dph APs revealed a marked decrease in maximum depolarization rate with increasing AP number, indicating a loss of Nav channel availability. This effect was noticeably less pronounced in older juveniles and adults (Fig. 6d-g, right). We also found a significant effect of age on the fractional loss in the maximum depolarization rate between the 1st and 2nd AP, with

significant differences across all pairwise comparisons during the +600 pA current injection (Fig. 6j). Together, these findings are consistent with the hypothesis that a large I_{NaR} component is important for preserving Nav channel availability during high frequency firing. These findings also provide evidence that the intrinsic excitability of RAPNs undergoes substantial developmental changes, in contrast with previous studies suggesting a lack of marked age-dependent changes⁴¹.

To facilitate long periods of whole cell recordings we routinely performed these current clamp recordings at room temperature (~24°C) because recordings did not last long at higher temperatures. However, the gating kinetics of ion channels are highly dependent on temperature, with Q_{10} values varying widely across different ion channels⁷⁰. We therefore performed an additional set of current clamp recordings in 20 dph and adult finch RAPNs at physiological body temperature (~40°C). Consistent with our room temperature recordings, we found significant differences between 20 dph and adult RA in both spikes number (Supplementary Fig. 6a-c) and IFF (Supplementary Fig. 6d) in response to injected current.

Navβ4 C-terminal peptide induces I_{NaR} and increases the excitability of juvenile RAPNs

To directly test whether Navβ4 is involved in generating an I_{NaR} in RAPNs, we dialyzed a 14-residue peptide derived from the Navβ4 C-terminus⁵¹ into RA neurons of 20 dph juveniles, shown previously to possess very low I_{NaR} (Fig. 4a-d; Fig. 5d-e). Consistent with a role for Navβ4 in conferring resurgent properties, intracellular dialysis of the β4-WT peptide elicited a large I_{NaR} within several minutes of achieving the whole-cell voltage clamp configuration (Fig. 7a₁-a₂). This effect was not seen with a scrambled version (β4-Scr, 100 μM) of this peptide (Fig. 7c;⁵¹). The average normalized I_{NaR} to I_{NaT} ratio in RA increased significantly with β4-WT (Fig. 7b) but not with β4-Scr (Fig. 7d) and the fold-change for this ratio differed significantly between cells exposed to β4-WT or β4-Scr (Fig. 7e). We conclude that the channel blocking carboxy-terminal domain of the Navβ4 protein is sufficient to elicit robust I_{NaR} in juvenile RAPNs.

We next examined whether dialyzing 20 dph RAPNs with the β4-WT peptide would make these neurons more excitable. Indeed, upon dialysis of the β4-WT in whole-cell current clamp recordings, we detected a prominent “spikelet” that developed in a time-dependent manner during the repolarization phase of the first AP elicited by a positive current injection (Fig. 7f). This “spikelet” appeared in 58% of cells (7/12 cells) dialyzed with β4-WT, but never in cells dialyzed with the β4-Scr (0/15 cells) (Fig. 7f and i). Exposure to the β4-WT broadened the AP half-width by 20% (Fig. 7h) and decreased the maximum rate of repolarization by an average of 17% (Supplementary Fig. 7a). Importantly, these effects were not seen with the β4-Scr (Fig. 7j-k; Supplementary Fig. 7b). If we treated “spikelets” as APs, the IFF recorded just after the start of dialysis was not different between groups, but after 15 minutes the IFF was significantly higher in cells that received the β4-WT than in cells that received β4-Scr (143 ± 21.2 Hz vs 84.6 ± 3.8 Hz; Mean $\pm$ SEM; Supplementary Fig. 7c-d).

***In silico* modulation of I_{NaR} alters the intrinsic excitability of RAPNs**

As a further test of the role of I_{NaR} in the regulation of excitability in RA, we modeled the I_{NaR} from male RAPNs using a previously published model available in the *SenseLab* database as a template⁷¹. Activation of this modeled I_{NaR} is contingent on brief periods of depolarization followed by hyperpolarization of the membrane potential (see Methods). The experimental data (Fig. 8a) matched the modeled I_{NaR} (Fig. 8b) with a close overlap of I-V curves (Fig. 8c) and similar decay kinetics at hyperpolarized potentials (<-20 mV; Fig. 8d).

We predicted that adding this *in silico* I_{NaR} via dynamic clamp would enhance aspects of excitability in juvenile RA, while subtracting it would depress excitability in adult RA. We tested these predictions by comparing the effects of delivering sequential +300 pA current injections at

baseline and during dynamic clamping of RAPNs. In 20 dph juveniles, we observed that addition of the modeled I_{NaR} during step-like current injections (Fig. 8e, blue) significantly depolarized the interspike periods, while reducing the interval between the first two spikes (Fig. 8e, red; Table 2) when compared to control (Fig. 8e, black; Table 2). This interspike depolarization was expected given the high input resistance of 20 dph RAPNs (422 MΩ; Table 1). As a result, the IFF increased significantly by 15-20% at all conductances tested (Fig. 8f). These results were reminiscent of our findings using the β4-WT peptide (Supplementary Fig. 7d). This supports a role for I_{NaR} in modulating inter-spike intervals. As with the β4-WT peptide (Fig. 7h and Supplementary Fig. 7a), we also noted an increase in the average AP half-width and a decrease in the average maximum repolarization rate (Table 2). Interestingly, the number of elicited APs decreased in a conductance-dependent manner with the injected *in silico* I_{NaR} (Table 2), perhaps due to a low density of voltage-gated K⁺ channels (Kvs) in juveniles that did not allow for a faster recovery of Navs from inactivation.

By contrast, when subtracting the modeled I_{NaR} in adults, we observed hyperpolarization of the membrane potential during the inter-spike periods (Table 3), coinciding with significant decreases in the IFF (5-10%) of the first two spikes when the subtracted *in silico* I_{NaR} conductance was ≥ 1 mS (Fig. 8g-h). Notably, this modulation did not significantly alter the total number of spikes produced during a 1 sec sweep (Table 3). Overall, these findings point to an important role of I_{NaR} in promoting the high-frequency firing of spikes in adult RAPNs.

Navβ4 mRNA, I_{NaR} and the excitability of RAPNs are sex dependent

Female zebra finches do not sing and show marked developmental atrophy in RA (Supplementary Fig. 5)^{56,57}. However, age-dependent changes in the intrinsic excitability of female RAPNs have not been previously examined. *In situ* hybridization for Navβ4 mRNA showed weak expression and no evidence of differential labeling in female RA compared to the surrounding arcopallium at 20 dph, 35 dph or 50 dph (Fig. 9a; refer to Fig. 3 for males). We note that female RA could be unequivocally identified with Nissl staining at the ages tested (Fig. 9b). Contrasting sharply with the marked age-dependent increases of males, we observed a small trend of increased Navβ4 expression after 20 dph (Fig. 9a and c) and a modest, significant increase in the proportion of Navβ4-expressing cells between 20 dph and older ages (Fig. 9a and d). Interestingly, these changes appeared to plateau at 35 dph (Fig. 9c and d). Based on this expression pattern, we predicted that the magnitude of I_{NaR} in female RA would be small across ages. Indeed, we detected a small I_{NaR} at 20 dph and a modest increase at 35 dph and no further increase at 50 dph (Fig. 9e-g). However, these trends were not statistically significant.

Whole-cell current clamp recordings of female RAPNs revealed spontaneous AP firing across all ages, but in contrast to males, there was little evidence of age-dependent changes in active or passive properties (Table 4). These neurons showed increased firing in response to current injections across ages (Fig. 10a-c, left, red traces), but unlike males (Fig. 6), we observed a progressive broadening and amplitude decrease when comparing the 1st, 2nd and 16th-21st AP at all ages examined (Fig. 10a-c, right). We also failed to detect significant age group differences in elicited spikes per sec, in the IFF, or in the change in the maximum depolarization rate ratio between the 2nd and 1st AP (Fig. 10g-i; Supplementary Fig. 8a-c). Phase plots revealed marked differences in rates of depolarization and hyperpolarization between male and female RAPNs at different ages. Specifically, while males had a progressive age-dependent increase in the rate of depolarization during spike trains, the shape of female phase plane plots remained largely unchanged throughout development (Fig. 10d-f). These marked sex differences could be clearly visualized on 3D graphs plotting the relationships between spikes per sec, IFF, and the change in the maximum depolarization rate (Fig. 10g-i). Specifically, while 20 dph males and females were tightly clustered together (Fig. 10g), a moderate separation appeared at 35 dph (Fig. 10h),

with the sexes occupying distinct clusters by 50 dph (Fig. 10i). Finally, we note the persistence of the double-hump shape of the female AP phase plot across ages (Fig. 10d-f), similar to that of 20 dph males (Fig. 6b; Fig. 10d, right), and in contrast to 35 dph and 50 dph males. The lack of change in this waveform suggests a persistently low density of Nav channels in the female RAPNs at the axon initial segment and in the soma-dendritic compartment⁴³.

Principal component analysis (PCA) applied to multiple spontaneous AP parameters from male and female RA neurons across ages provided quantitative support and further insights into these marked developmental sex differences. PCA1, which accounted for 53% of the total variance, segregated males but not females according to age, with 50 dph males localizing to the right quadrants, whereas younger males and all females from all age groups localized mostly to the left quadrants (Supplementary Fig. 9, x-axis). Interestingly, PCA1 accounted for >80% of the variance of AP half-width and the maximum rates of depolarization and repolarization, half-width showing a strong negative correlation with the other two parameters. PCA2 and PCA3 accounted respectively for 20%, and 17% of the total variance. PCA2 predominantly accounted for the variance of the AP amplitude and threshold, which showed a strong negative correlation, and PCA3 almost exclusively accounted for the variance in the spontaneous firing frequency, but neither PCA2 (Supplementary Fig. 9, y-axis) nor PCA3 (not shown) helped segregate the data by age groups.

Discussion

Our results reveal that AP waveforms in mature RAPNs have: 1) low spike thresholds, 2) short half-widths, 3) non-adapting amplitudes at high firing frequencies, and 4) large maximum depolarization and repolarization rates (Table 1). These properties strongly resemble those of large motor L5PNs in mammals, and emerge progressively in an age-dependent manner that parallels the known critical period of vocal learning in male, but not female, finches. These changes show a strong correlation with developmental changes in Navβ4 expression and I_{NaR} . Experiments utilizing dynamic clamp and Navβ4 peptides in juveniles support the conclusion that I_{NaR} provides an important contribution to the intrinsic excitability of RAPNs. Besides presenting strong evidence linking an I_{NaR} to Navβ4 expression, this work provides insight into the molecular requirements of upper motor neurons that drive spectrally complex, learned vocalizations.

RAPNs operate as pyramidal-like motor neurons

Precise movements and motor learning in mammals requires the coordinated fast activation and suppression of muscles via output signaling from L5PNs in M1 motor cortex¹. Likewise, song production in the zebra finch requires fast and precise signaling from RAPNs to activate and suppress syringeal musculature³¹. Despite circuit and gene expression evidence suggesting these neuron types are analogs^{16,17,19}, functional evidence at the single neuron biophysical level was previously lacking.

Our study reveals striking similarities between the intrinsic excitability of RAPNs and the “regular spiking” L5PNs found in motor cortex^{47,72,73}. During periods of silence, adult RAPNs exhibit periodic spontaneous firing^{24,39,40} that mirrors that of rat L5PNs in M1^{72,73}. Like L5PNs²⁵, RAPNs have an I_h -mediated sag⁴⁰ and a persistent sodium current that likely contributes to this spontaneous periodic spiking in both cell types. These currents, together with I_{NaR} , may facilitate the depolarizing after potential that follows the AP spike, thus setting the periodicity of the spontaneous spiking (Figure 1c). An additional hallmark of L5PNs is an AP phase plot with a biphasic upstroke phase showing a distinct axon initial segment component with an abrupt “kink” followed by a soma-dendritic component⁴³. This closely mirrors our results in Fig. 1e. Voltage-

clamp recordings from nucleated patches of L5PNs also reveal a fast non-linear activation of I_{NaT} with a short onset delay⁶⁵. Moreover, the developmental profile of RAPNs closely resembles those of maturing rat L5PNs, including narrowing of spike waveforms and increases in spike firing frequency²⁶.

Narrow action potential spikes may facilitate temporal coding

Pyramidal neurons with multiple dendritic branches and a high density of Na^+ channels at a distal axonal spike initiation site have decreased effective time constants and exhibit rapid-onset spikes with low thresholds⁷⁴⁻⁷⁶. Narrow spikes with rapid onset allow phase-locking to high input stimulation frequencies. Accordingly, large human cortical neurons encode higher bandwidths of synaptic input information with low-threshold, rapid-onset spikes compared to smaller mouse cortical neurons that have higher thresholds and slower spike initiation⁷⁷. Our results suggest that a large I_{NaT} generates the rapid upstroke of the narrow AP in the adult RAPNs, making them well “engineered” to integrate and encode bursts of high-frequency synaptic inputs at their multiple spiny dendrites²⁴.

Interestingly, L5PNs in the M1 cortex of awake macaques have large-caliber axons and exhibit narrow AP spikes (0.26 ms)⁹ with fast conduction velocities (~80 to 90 m/s)¹⁰. Narrow spikes (half-width = 0.42 ms) are also present in large L5PNs in cat motor cortex¹¹. Our results demonstrate a similarly fast activation of I_{NaT} in RAPNs that produces a rapid AP upstroke (Fig. 5g), coupled to a fast I_{NaT} inactivation that helps to produce a narrow AP half-width (0.18 ms at 40°C; Sup. Fig. 2a-c). We also note that while RA neurons in the finch⁷⁸ and Betz-type L5PNs in primate cortex¹⁰ express Kv3.1, expression of this channel is notably absent in rodent L5PNs¹⁰. The fact that Kv3.1 currents are associated with fast spiking neurons with narrow APs in several brain areas⁷⁹ may partially explain differences in the spike properties of upper motor neurons in different species. Finally, while our data indicates narrower AP spikes in RA than those previously reported^{24,39,40}, this may be due to previous studies using lower temperatures, neurobiotin cell fills⁸⁰, and/or higher external divalent ion concentrations⁸¹. Taken together, our results thus reveal specialized intrinsic properties of RAPNs that contrast starkly with those in pre-motor HVC neurons⁸² and make them well suited to produce a precise temporal code for fine motor control³¹.

Fast I_{NaT} inactivation and energy efficient spikes

Prolonged burst firing of non-adapting spikes at high frequencies greatly increases the bandwidth of information processing for RAPNs during song production. This requires a constant operation of Na^+/K^+ -ATPase pumps, which is energetically expensive. Thus, it is not surprising that RA exhibits dense cytochrome C staining³⁴. However, energy demands can be kept within reasonable bounds if the narrow spikes of RAPNs are energy efficient. For example, L5PNs exhibit energy-efficient narrow spikes with reliable spike initiation⁸³ and smaller amplitudes at 37°C⁴⁶. Fast I_{NaT} inactivation kinetics is key for energy efficiency because it allows for non-overlapping Na^+ and K^+ currents during the upstroke and downstroke of the AP⁸⁴⁻⁸⁷. The fast I_{NaT} inactivation of adult RAPNs that we report here (Fig. 5) may thus help to reduce energy consumption. We also note that unlike males, the comparatively smaller female RA, with PN that fail to fire at high frequencies, display less cytochrome C staining³⁴.

Developmental changes in RAPN intrinsic excitability

Previous studies have attributed major *in vivo* changes in RA activity during the vocal learning period primarily to changes in the synaptic strength of inputs from HVC and LMAN^{32,41,81,88}. However, we observed significant changes between 35 and 50 dph juveniles in several intrinsic excitability features of RAPNs, including input resistance, spike halfwidth, maximum depolarization and repolarization rates, and spike number during high frequency firing (Fig. 6 and Table 1). Furthermore, while the spike frequency during positive current injections reportedly did

not change between 50 dph juveniles and adults⁴¹, we found significant changes in spike threshold and waveform between these ages. We therefore conclude that in addition to changes in synaptic plasticity, significant changes to intrinsic neuronal excitability may also contribute to the developmental increase in burst firing and decrease in spike variability of RAPNs. Importantly, we also show strong developmental upregulation of both Nav β 4 and Nav β 1 in addition to downregulation of Nav β 3 in RA during the critical period of vocal learning. This change in subunit expression strongly correlated with the appearance of a larger I_{NaT} and I_{NaR} , leftward shifts in I_{NaT} activation, and faster I_{NaT} inactivation, all properties thought to facilitate high frequency AP firing. These observations support the notion that developmental changes in RAPN excitability might be linked to changes in modulatory β subunit expression.

Auxiliary Nav β subunits help to shape spike waveforms in RAPNs

Adult male finches show higher expression of Nav1.6 (SCN8A) in RA compared to the surrounding arcopallium⁴⁹. Importantly, this channel carries the majority of I_{NaR} in a number of cell types⁵². We suggest that a combination of fast inactivation mediated by the Nav1.6 α subunit (hinged-lid mechanism), together with the blocking peptide of the covalently bound Nav β 4 may greatly accelerate inactivation kinetics seen in Figure 5h. In addition, the Nav β 4 blocking peptide is thought to protect Nav channels from entering a long-lived inactivation state⁵². A large surplus of Nav channels available for opening is critical for limiting cumulative inactivation, thus promoting reliable, high frequency AP firing with little amplitude attenuation⁸⁹. Interestingly, coexpression of Nav1.6 and Nav β 4 in HEK cells causes a hyperpolarizing shift in activation (~ 8 mV)⁹⁰ similar to what we observed (Fig. 5f; a ~ 10 mV hyperpolarizing shift of the Na⁺ current IV curve from 20 dph to adult). Without a high density of available Nav channels, RAPNs would exhibit smaller spike amplitudes, prolonged interspike intervals and AP failures during high frequency firing⁸⁹ as seen in 20 dph juveniles (Fig. 6) and in arcopallial neurons outside RA in adults (Fig. 2).

Adult RA also shows high expression of Nav β 1, which bind Nav α subunit non-covalently⁹¹. In mammals Nav β 1 promotes the localization of Nav1.6 to the axon initial segment of L5PNs⁹², and contributes to I_{NaR} in cerebellar granule cells⁹³. Nav1.6 exhibits fast inactivation^{94,95} and co-expression with Nav β 1 greatly accelerates recovery from inactivation, as well as expression of a small persistent current ($\sim 5\%$ of peak current at +20 mV)⁹⁵. It thus seems likely that high expression of Nav1.6, combined with Nav β 1 and Nav β 4 may contribute to generating large Na⁺ currents with fast activation and inactivation kinetics in RAPNs^{90,94}. Of note, Nav β 2 has not been linked to I_{NaR} and is not differentially expressed across the arcopallium⁴⁹.

Nav β 4 peptide and I_{NaR} promote excitability in RAPNs

Although Nav β 4 has been linked to I_{NaR} and intrinsic excitability in a number of cell-types^{51,67,96,97}, such a link has also been disputed in recent studies where elimination of the Nav β 4 gene failed to eliminate this current in cerebellar purkinje neurons^{98,99}. However, the knockout was constitutive, and alternative compensatory mechanisms may have enabled this I_{NaR} current⁹⁹. In our study, the β 4-WT peptide was sufficient to increase I_{NaR} and the IFF of RAPNs from pre-song juvenile finches in a time dependent manner, providing strong evidence of a causal link between Nav β 4 and these excitable properties. Most recordings resulted in a second 'spikelet' before the first AP had fully repolarized, in some cases resembling the after-depolarization seen in adult RAPNs (Fig. 1c). A similar effect is also seen at the rodent calyx of Held when this peptide is dialyzed into immature nerve terminals⁶⁷. Fast AP repolarization rates are absent in 20 dph finches (Table 1), which may explain why we did not see a time dependent increase in the number of spikes in these peptide experiments. We suggest that with a faster AP repolarization rate, these "spikelets" would have produced full blown spikes that would have more closely resembled the mature AP waveform and firing.

The dynamic clamp approach we used to further study the role of I_{NaR} in RAPN spiking revealed that the addition of an *in silico* I_{NaR} increases the instantaneous firing frequency of 20 dph RAPNs. Conversely, subtraction of I_{NaR} significantly decreased the instantaneous firing frequency of adult RAPNs (Fig. 8). Because the addition or subtraction of a modeled I_{NaR} has no direct effect on Nav channel availability, like a pharmacological peptide blocker would, these results provide strong evidence that I_{NaR} alone is sufficient to enhance the firing frequency of RAPNs.

Large I_{NaR} and fast I_{NaT} inactivation kinetics are also observed in the mature calyx of Held nerve terminal^{67,100}, which exhibits narrow AP spikes that can fire at high frequencies (up to 1 kHz;¹⁰¹). Moreover, I_{NaR} generates a depolarizing after potential at the calyx of Held once the spike downstroke has terminated⁶⁷, similar to that seen in RAPN spikes (Figure 1c). Interestingly, cells in layer 5 of mouse primary motor cortex (M1) also express Navβ4 mRNA¹⁰² and Navβ4 protein is specifically expressed at the axon initial segment of L5PNs of the mouse somatosensory cortex¹⁰³, although it is currently unknown whether L5PNs have I_{NaR} .

Sex differences in the intrinsic excitability of maturing RAPNs

Although largely unexplored, defining sex differences in the excitability of zebra finch song nuclei may help to identify critical requirements for neurons that control learned vocalizations. Interestingly, the developmental changes in Navβ4 expression, I_{NaR} , spike waveforms and firing rates of RAPNs seen in males were absent in females, which do not sing (Figure 10). These observations provide further support for Navβ4 expression underlying I_{NaR} , and thus contributing to the intrinsic excitability properties of RAPNs. While we acknowledge that a number of other factors likely determine the lack of female vocal learning^{56,104}, we suggest that the lack of maturation in the intrinsic properties of RAPNs could contribute to females being unable to produce complex, learned vocalizations.

Conclusions

Our results point to a key piece of “hardware” that allows adult male RAPNs to function as precise and reliable generators of fast spiking: High expression of Navβ4, likely coupled with Navβ1 and Nav1.6α subunits, to produce a large I_{NaR} and an I_{NaT} with fast activation and inactivation. These features lower AP threshold and allow robust rapid-onset APs with large non-adapting amplitudes⁷⁶. Possibly in concert with Kv3.1 channels^{10,79}, this combination of Navα/β subunits narrows the spike half-width and enables fail-safe fast spiking activity to emerge during the critical song learning period. By contrast, RAPNs in female and male juveniles express high levels of Navβ3, do not have a large I_{NaR} , and cannot spike at high frequencies. Finally, we note that a subclass of large Betz-type L5PNs in primate motor cortex^{9,10} also exhibit narrow spikes that may fine tune them for rapid and precise motor control³. We thus propose that the specialized “hardware” of RAPNs facilitate their precise *in vivo* spike burst firing that is required for activation of lower motor neurons and contraction of the superfast syrinx muscles involved in singing³³.

Methods

Animal subjects: All of the work described in this study was approved OHSU’s Institutional Animal Care and Use Committee and is in accordance with NIH guidelines. Zebra finches (*Taeniopygia guttata*) were obtained from our own breeding colony or purchased from local breeders. The ages of birds in the 20, 35, and 50 days post hatch (dph) groups were established based on the date the first egg hatched, and thus the birds were sacrificed within ± 2 days of the target age. Birds older than 120 dph were considered adults. On the day of the experiment, all of the birds with the exception of 20 dph birds, were removed from the colony at lights-on (9 AM PST) and housed for ~1 hr in an acoustic isolation chamber to minimize the potential confounds of singing and non-

specific auditory stimulation on gene expression, noting that social isolation can also alter neurogenomic state¹⁰⁵. The 20 dph finches were removed directly from the colony, and not isolated, so as to minimize stress on newly fledged birds. The sex of 20 and 35 dph birds could usually be distinguished by plumage, however we routinely confirmed the sex of individuals by gonadal inspection. Birds were sacrificed by decapitation and their brains removed. For electrophysiology experiments brains were bisected along the midline, immersed in ice-cold cutting solution, and processed as described below. For a subset of these brains (N=4 per sex per age group), we reserved one hemisphere for electrophysiology experiments and the other was placed in a plastic mold, covered with ice-cold Tissue-Tek OCT (Sakura-Finetek; Torrance, CA), and frozen in a dry ice/isopropanol slurry for *in situ* hybridization. The use of right vs. left hemispheres was balanced to account for any hemispheric differences in electrophysiology or gene expression. For the remaining brains, both sides were used for electrophysiological recordings.

In situ hybridization: To compare mRNA expression levels for *SCN3B* and *SCN4B* across developmental ages and between sexes, brains sections (thickness = 10 μ m) were cut on a cryostat onto glass microscope slides (Superfrost plus; Fisher Scientific) and stored at -80°C. For each brain, a set of slides consisting of every 10th slide was fixed and stained for Nissl using an established protocol. Slides were examined under dark- and bright-field microscopy to identify sections containing the core region of nucleus RA, i.e. the sections where RA appears largest. *In situ* hybridizations (ISH) were conducted using established protocols¹⁰⁶ and were performed in separate batches for each gene to standardize fixation and hybridization conditions. Briefly, slides were hybridized under pre-optimized conditions with DIG-labeled riboprobes synthesized from BSSHII digested cDNA clones obtained from the ESTIMA: Songbird clone collection¹⁰⁷, corresponding to GenBank entries FE734016 (*SCN3B*; aka Nav β 3), FE730991 (*SCN4B*; Nav β 4), and DV957065 (*SCN1B*; Nav β 1). After hybridization, slides were washed, blocked, incubated with alkaline phosphatase conjugated anti-DIG antibody (Roche, 1:600), and developed overnight in BCIP/NBT chromagen (Perkin Elmer). Slides were coverslipped with VectaMount (Vector) permanent mounting medium, and then digitally photographed at 10X under brightfield illumination with a Lumina HR camera mounted on a Nikon E600 microscope using standardized filter and camera settings. Images were stored as Tiff files and analyzed further using the FIJI distribution of ImageJ¹⁰⁸. We note that high-resolution images of *SCN1B*, *SCN3B*, and *SCN4B* expression in the adult male zebra finch brain are available on the Zebra Finch Expression Brain Expression Atlas (ZEBra; www.zebrafinchatlas.org).

Image Analysis and Expression Quantification: For each image, we quantified both expression levels based on labeling intensity (i.e. average pixel intensity) and the number of cells expressing mRNA per unit area. Since the density of cells in RA is known to change during development and to vary by sex⁵⁶, we first estimated the number of neurons in RA and caudal to RA for each bird by placing a 200 x 200 μ m window over target areas in the images of the Nissl-stained sections containing the largest RA per bird. We next measured the average pixel intensity (scale: 0-256) in an identical 200 x 200 μ m window placed over each target area in the images of hybridized sections adjacent to the Nissl-stained sections. From the values we then subtracted an average background level measured over an adjacent control area in the intermediate arcopallium that was deemed to have no mRNA expression. Finally, we divided the background corrected pixel intensity value by the number of Nissl-counted cells in order to obtain a measurement of the average pixel intensity per cell. We also quantified the number of labeled cells in each arcopallial region by first establishing a threshold of expression 2.5X above the background level. Standard binary filters were applied and the FIJI 'Analyze Particles' algorithm was used to count the number

of labeled cells per 200 μm^2 . This value was further normalized for comparisons across different ages and sex by dividing by the number of Nissl-labeled cells from the adjacent section.

Slice Preparation for Electrophysiology Experiments: Sagittal (200 μm thick) and frontal (150 μm thick) slices were cut on a vibratome slicer (VT1000, Leica) in an ice-cold cutting solution containing (in mM): 119 NaCl, 2.5 KCl, 8 MgSO_4 , 16.2 NaHCO_3 , 10 HEPES, 1 NaH_2PO_4 , 0.5 CaCl_2 , 11 D-Glucose, 35 Sucrose pH 7.3-7.4 when bubbled with carbogen (95% O_2 , 5% CO_2 ; osmolarity ~ 330 -340 mOsm). Slices were then transferred to an incubation chamber containing artificial cerebral spinal fluid (aCSF) with (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO_4 , 26.2 NaHCO_3 , 1 NaH_2PO_4 , 1.5 CaCl_2 , 11 D-Glucose, 35 Sucrose pH 7.3-7.4 when bubbled with carbogen (95% O_2 , 5% CO_2 ; osmolarity ~ 330 -340 mOsm) for 10 min at 37°C, followed by a room temperature incubation for ~ 30 min prior to start of electrophysiology experiments. For low-sodium aCSF experiments (see Fig. 5), 119 NaCl mM extracellular sodium was replaced with 30 mM NaCl and 90 mM NMDG, the pH was adjusted to 7.4 with HCl, and the osmolarity was adjusted to ~ 330 -340 with sucrose.

Patch Clamp Electrophysiology: RA could be readily visualized in males and females of all ages via infra-red differential interference contrast microscopy (IR-DIC) (Supplementary Fig. 5). Whole-cell patch-clamp recordings were performed at room temperature ($\sim 24^\circ\text{C}$) unless otherwise indicated. For experiments performed at 40°C , the bath solution was warmed using an in-line heater (Warner Instruments, Hamden, CT). The temperature for these experiments varied up to $\pm 2^\circ\text{C}$. Slices were perfused with carbogen-bubbled aCSF (1-2ml/min) and neurons were visualized with a IR-DIC microscope (Zeiss Examiner.A1) under a 40x water immersion lens coupled to a CCD camera (Q-Click; Q-imaging, Surrey, BC, Canada). Whole-cell voltage- and current-clamp recordings were made using a HEKA EPC-10/2 amplifier controlled by Patchmaster software (HEKA, Ludwigshafen/Rhein, Germany). Data were acquired at 40 kHz and low-pass filtered at 2.9 kHz. Patch pipettes were pulled from standard borosilicate capillary glass (WPI, Sarasota, FL) with a P97 puller (Sutter Instruments, Novato, CA). All recording pipettes had a 3.0 to 6.0 M Ω open-tip resistance in the bath solution. Electrophysiology data were analyzed off-line using custom written routines in IGOR Pro (WaveMetrics, Lake Oswego, OR).

For voltage clamp (VC) recordings, intracellular solutions contained the following (in mM): 142.5 Cs-Gluconate, 11 CsCl, 5.5 Na_2 -phosphocreatine, 10.9 HEPES, 5.5 EGTA, 10.9 TEA-Cl, 4.2 Mg-ATP, and 0.545 GTP, pH adjusted to 7.3 with CsOH, ~ 330 -340 mOsm. Alexafluor 488 hydrazide (ThermoFisher; 10 μM) was added to this solution to aid in cell-type morphology discrimination via epifluorescent microscopy (X-Cite series 120, Excelitas Technologies, Waltham, MA). In recordings where the series resistance (R_s) was compensated to 5 M Ω , the average uncompensated $R_s = 10.2 \pm 0.5$ M Ω ($n=66$ cells for male/female). In recordings in Fig. 5 where R_s was compensated to 1 M Ω , the uncompensated $R_s = 7.2 \pm 0.5$ M Ω ($n=8$ for males at 20 dph and adults). In order to isolate Na^+ currents, slices were exposed to bath applied CdCl_2 (100 μM), 4-AP (100 μM), Picrotoxin (100 μM), TEA (10 mM), CNQX (10 μM), and APV (100 μM) for ~ 5 min prior to running voltage clamp protocols. After protocols were applied, tetrodotoxin (TTX 1 μM) was bath applied and the same voltage-clamp protocols were repeated after Na^+ currents were eliminated. Na^+ currents were isolated by subtracting the TTX-insensitive current traces from the initial traces. Capacitive currents generated during voltage-clamp recordings were eliminated by P/4 subtraction. Recordings were not corrected for a calculated liquid junction potential of 12 mV.

For current clamp recordings (CC), intracellular solutions contained (in mM): 142.5 K-Gluconate, 21.9 KCl, 5.5 Na_2 -phosphocreatine, 10.9 HEPES, 5.5 EGTA, 4.2 Mg-ATP, 0.545 GTP, and 10 μM Alexafluor 488 hydrazide, pH adjusted to 7.3 with KOH, ~ 330 -340 mOsm. Synaptic currents were blocked by bath applying Picrotoxin (100 μM), APV (100 μM), and CNQX (10 μM) (Tocris

Bioscience) for ~3 min prior to all recordings. To initiate CC recordings, we first established a gigaohm seal in the VC configuration, set the pipette capacitance compensation (C-fast), and then set the voltage command to -70 mV. We then applied negative pressure to break into the cell. Once stable, we switched to the fast CC configuration. Experiments in CC were carried out within a 15-min period. We noted that the resting membrane potential tended to hyperpolarize to the same degree (~10 mV) in both juveniles and adults during these CC recordings¹⁰⁹. Recordings in which the resting membrane potential deviated by > 15 mV were discarded. We note that recordings were not corrected for a calculated liquid junction potential of 9 mV.

Estimated current clamp measurements of membrane capacitance (C_m) were calculated from the measured membrane time constant (τ_m) and input resistance (R_{in}) using the equation:

$$\tau_m(ms) = R_{in}(M\Omega) \cdot C_m(pF)$$

Estimated peak Na^+ current (I_{Na}) activated during the upstroke of the action potential (AP) was calculated from the capacitive membrane current using the equation:

$$max. I_{Na} (pA) = C_m (pF) \cdot max. dV_m/dt (V/s)$$

where peak depolarization rate is $max. dV_m/dt$ (see Vaaga et al. (2020)⁶⁹ and Yu et al. (2012)⁴⁶).

Dialyzed Peptides: Mouse $\beta 4$ -WT (KKLITFILKKTREK) and $\beta 4$ -Scr (KIKIRFKTKTLELK) peptides (as previously described in Grieco et al. 2005⁵¹) were synthesized by Thermofisher scientific at 98% purity and diluted to 100 μ m in internal solutions for CC and VC experiments. The mouse peptide was used because the C-terminal domain of Nav $\beta 4$ is highly conserved across vertebrates and found to be readily soluble in our internal solutions⁵³.

Dynamic Clamp parameters: Dynamic clamp was implemented in a Hewlett Packard PC (Z600 workstation) running Windows 10 software¹¹⁰. Voltage values were read and calculated currents outputted through a National Instruments DAQ board (PCIe-6321) controlled by Igor Pro 8 using the NIDAQ Tolls MX package (Wavemetrics). We utilized a model deposited in ModelDB within the *Senselab* database (see accession number 127021)^{71,111}. The I_{NaR} current was modeled as a single gate relying into two particles: one for activating (s) and one for inactivating (f). Each of these particles has a separate probability for opening (i.e. going to a permissive state: α) and closing (i.e. going to a non-permissive state: β). In order to match the kinetics of the model to zebra finch I_{NaR} currents, we made minor changes to the equations describing the rate constants in the original model⁷¹. We adjusted two parameters of the s particle rate constants: (1) an exponential term denominator ($K\alpha_s$ from -6.82 to -1.0) and (2) the magnitude of the multiplying term β rate constant ($A\beta_s$ from 0.0156 to 0.05).

To calculate the *in silico* current, we used a set of first order Hodgkin-Huxley type equations:

$$I_{NaR} = g_{NaR} * s * f * (V - E_{NaR}) \quad (1)$$

$$\tau_x = \frac{1}{(\alpha_x + \beta_x)} \quad (2)$$

$$x^\infty = \alpha_x * \tau_x \quad (3)$$

$$\frac{dx}{dt} = \frac{x^\infty - x}{\tau_x} \quad (4)$$

Where x represents either the s or f gating particles. The differential equation (4) was solved using a first order Euler method¹¹² with a time step of 0.03 ms. The g_{Nar} represents the maximum conductance (in mS) and the calculated reversal potential (E_{Nar}) was +67 mV. All neurons recorded were kept approximately at -70 mV throughout the current clamp recordings. To trigger action potentials, a step current injection of +300 pA (relative to the holding current) was applied for 1 sec. A complete list of the numeric parameters and equations is available in ModelDB (modeldb.yale.edu/127021)^{71,111}.

Statistical Data Analysis and Curve Fitting: Data were analyzed off-line using IgorPro software (Wave-metrics). Statistical analyses were performed using Prism 4.0 (GraphPad). Specific statistical tests and outcomes for each analysis performed are indicated in the respective Figure Legends and Tables. Means and SE are reported, unless otherwise noted.

Figures and Legends:

Figure 1. Intrinsic excitable properties of RA projection neurons (RAPNs) in adult male zebra finches at room and physiological temperatures. **a**. Top: Zebra finch vocal control circuitry; nuclei and connections of the direct vocal-motor pathway shown in black, RA and its descending projections highlighted in red, other nuclei and connections omitted for clarity. Dashed line represents the dorsal boundary of the arcopallium. HVC is a proper name; RA, robust nucleus of the arcopallium; nXIIIts, tracheosyringeal division of the hypoglossal nerve nucleus; RAM, nucleus retroambigualis medialis. Bottom: Diagram depicting upper (red) and lower (blue) motor neurons of the descending motor pathway in zebra finch, a songbird (left), and mammals (right). **b**. Whole cell current clamp recording of spontaneous APs during 1 sec. **c**. Spontaneous AP from a different cell than (b), arrow indicates after-depolarization. **d**. Representative spontaneous AP from (b; black) with overlay of spontaneous APs from an additional 15 RAPNs (pink); values for the indicated parameters are presented in Table 1. **e**. Phase plane plots of the AP in (d; black) and from another 15 RAPNs (pink, same cells as in d); indicated are the presumed contributions of the axonal initial segment (AIS) and somato-dendritic components to the biphasic depolarization component in the phase plane plot. **f**. Whole cell current clamp recording of spontaneous APs during 1 sec in a RAPN at physiological temperature (40°C). **g**. Representative spontaneous AP recorded at physiological temperature (40°C; black) with overlay of a spontaneous AP recorded at room temperature (24°C; blue). **h**. Overlaid phase plane plots of the spontaneous APs from g. **i**. Representative AP train elicited by a 1 sec 500 pA current injection at 40°C; the corresponding plot of instantaneous firing frequency as function of time is shown at the top. **j**. First two APs from (i). **k**. Overlay of the phase plane plots from the two APs in (j).

Figure 2. Expression of Navβ4 and β3 mRNAs, I_{NaR} and intrinsic excitability in the arcopallium of adult male zebra finches. **a.** Anatomical drawing indicating the position of RA within the arcopallium in a parasagittal section; distance from the midline indicated; the shaded area represents the region outside RA that is compared with recordings within RA. Arco., arcopallium; Nido., Nidopallium; OM, occipitomesencephalic tract; St, striatum; TeO, Optic tectum. orientation: dorsal is up and anterior to the left. **b.** *In situ* hybridization images show high Navβ4 expression (left) and lack of Navβ3 expression (right) in RA (indicated by arrowheads). **c.** Representative examples of I_{NaT} and I_{NaR} elicited in an RAPN by the voltage clamp protocol at the top. I_{NaT} was elicited via a 10 ms step to +30 mV followed by test potentials to +15 (pink), 0 (grey), -15 (red), -30 (blue), -45 (purple), -60 (green) and -75 mV (black) to elicit I_{NaR} . The cell was held at -90 mV during a 2 sec intersweep interval. Inset: Image of a patch pipette filled with fluorescent dye for RA recordings. **d.** Detail views of example I_{NaT} and I_{NaR} elicited in an RAPN (black) and in a neuron outside RA (grey) by the voltage clamp protocol on the top. The I_{NaT} peaks have been truncated. **e.** Average I-V curves for the peak I_{NaR} in RAPNs (black) and in neurons outside RA (grey) (two-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(6, 146) = 27.00$, $N = 12$ RAPNs and 11 neurons outside RA). **f.** Average I-V curves after normalization of the peak I_{NaR} to the peak I_{NaT} measured in a given sweep then averaged across cells (two-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(6, 147) = 29.36$, $N = 12$ RAPNs and 11 neurons outside RA). **g.** Overlay of AP trains elicited by a 1 sec 500 pA current injection in an RAPN and in a neuron outside RA. **h.** The first 3 APs from the AP trains shown in (g). **i.** Average elicited spikes/sec as a function of current injected (two-way ANOVA with Tukey's post-hoc; $P < 0.0001$, $F(6, 160) = 22.79$, $N = 18$ RAPNs and 12 neurons outside RA). **j.** Average instantaneous firing frequencies measured as a function of current injected (two-way ANOVA with Tukey's post-hoc; $P < 0.0001$, $F(5, 138) = 5.647$, $N = 18$ RAPNs and 12 neurons outside RA). For data in e-f and i-j, * = $P \leq 0.05$ by post-hoc analyses.

Figure 3. Age-dependent changes in expression of Navβ3 and 4 mRNAs in the arcopallium of male zebra finches across ages. **a-d.** Representative *in situ* hybridization images for Navβ4 mRNA within RA across ages; black arrowheads indicate RA borders. **e-h.** Representative *in situ* hybridization images for Navβ3 mRNA within RA across ages. Black arrowheads indicate RA borders. **i.** Comparison of Navβ4 expression levels (normalized optical density) across age groups within RA (black) and in an arcopallial region of equal size outside RA (grey). Stars depict significant differences in RA (one-way ANOVA with Tukey's post hoc; $P = 0.005$, $F(3, 12) = 7.416$, $N = 4$ males per age). **j.** Comparison of the proportions of Navβ4-expressing cells across age groups within RA (black) and in an arcopallial region of equal size outside RA (grey). Stars depict significant age differences in RA (one-way ANOVA with Tukey's post hoc; $P = 0.001$, $F(3, 12) = 10.79$, $N = 4$ males per group). **k.** Comparison of Navβ3 expression level (normalized optical density) across age groups within RA (black) and in an arcopallial region of equal size outside RA (grey). Stars depict significant differences in RA (one-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(3, 12) = 73.43$, $N = 4$ males per group). **l.** Comparison of the proportions of Navβ3-expressing cells across age groups within RA (black) and in an arcopallial region of equal size outside RA (grey). Stars depict significant age differences in RA (two-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(3, 12) = 90.96$, $N = 4$ males per group). In l-l: * = $P \leq 0.05$, ** = $P \leq 0.01$, **** = $P \leq 0.0001$.

Figure 4. Age-dependent changes in I_{NaR} in RAPNs of male zebra finches. **a.** Examples of I_{NaT} and I_{NaR} elicited across ages by the voltage clamp protocol shown at the top. The large I_{NaT} peaks have been truncated; the color code is the same as in Fig. 2A. **b.** Representative currents from RAPNs at each age group during the -30 mV test potential shown at the top. The large I_{NaT} peaks have been truncated. **c.** Average I-V curves for the peak I_{NaR} in RAPNs from each age group (two-way ANOVA with Tukey's post hoc comparisons at the -30 mV test pulse; $P < 0.0001$, $F(18, 280) = 16.98$, $N(\text{cells/age}) = 10/20 \text{ dph}$, $12/35 \text{ dph}$, $10/50 \text{ dph}$ and $12/\text{adults}$). **d.** Average I-V curves after normalization of the peak I_{NaR} to the peak I_{NaT} measured in a given sweep then averaged across cells (two-way ANOVA with Tukey's post hoc comparisons at the -30 mV test pulse; $P < 0.0001$, $F(18, 280) = 23.72$, $N(\text{cells/age}) = 10/20 \text{ dph}$, $12/35 \text{ dph}$, $10/50 \text{ dph}$ and $12/\text{adults}$). **e.** Linear regression between average peak I_{NaR} values at the -30 mV test potential across ages and the average $\text{Nav}\beta 4$ expression level in RA. **f.** Linear regression between average peak I_{NaR} values at the -30 mV test potential across ages and the proportion of $\text{Nav}\beta 4$ -expressing cells in RA. For views of recorded slices across ages, see Supplementary Figure 5a-c.

Figure 5. Recording I_{NaT} and I_{NaR} in RAPNs with reduced extracellular Na^+ in frontal sections from 20 dph and adult male zebra finches. **a.** Myelin-stained parasagittal section containing nucleus RA (from *zebrafinchatlas.org*¹¹³; dorsal is up and anterior to the left). **b.** Detailed view of area shown in the dashed red rectangle in (a) depicts myelinated fiber bundles that leave RA, run rostrally, and enter the OM (occipitomesencephalic tract). **c.** Schematic drawing depicts how generating frontal sections (e.g. dashed line with scissors) transect the axonal fibers closer to the somata of RAPNs (black dots in RA), resulting in shorter initial axonal segments of RAPNs than in parasagittal slices. **d.** Overlay of representative currents from 20 dph (green) and adult (black) finches during a -30 mV test potential (top). Green arrow points to the 20 dph I_{NaT} peak. I_{NaR} has been enlarged in the inset. **e.** I_{NaR}/I_{NaT} measured at -30 mV in 20 dph and adult finches (N (cells/age) = 3/20 dph and 5/adult finches; Student's t -test). **f.** Average I - V relationship for I_{NaT} in 20 dph (green) and adult (black) finches. Curve fits for the I - V shown in the red lines. **g.** Curve fit for the current activation shown (red) for averaged I_{NaT} from 20 dph (green) and adult (black) finches where N is the apparent number of transitions in a Markov chain model (equation shown in Methods), τ is the time constant of activation. Scaled I_{NaT} from individual neurons used for averaging shown for adult (grey) and 20 dph (light green) finches. Arrows point to current onsets measured as the first point deviating from a line fit to the pre-stimulus baseline current. **h.** Representative I_{NaT} scaled to the peak from 20 dph and adult; the decay phases have been fitted with single exponentials (red) which overlay the respective traces. **i.** Comparison of the time constant of decay for I_{NaT} in 20 dph and adult finches (N (cells/age) = 3/20 dph and 5/adult males; Student's t -test).

Figure 6. Age-dependent changes in intrinsic excitable properties of RAPNs in male zebra finches. **a.** Overlay of representative spontaneous APs recorded across ages. **b.** Overlay of phase plane plots derived from the APs shown in (a). **c.** Enlarged view of the area highlighted in grey in (b). **d-g.** Left: Representative AP trains elicited by 1 sec 500 pA current injection in RAPNs across ages representative of vocal development stages. Center: APs #1, 2 and 16-21 from the traces on the left aligned at the peaks for each age group. Right: Phase plane plots from APs #1, 2, and 16-21 plotted at the same scale for all age groups. **h.** Average number of spikes produced during 1 sec as a function of current injected for each age group (two-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(18, 494) = 4.189$, $N(\text{cells/age}) = 18/20 \text{ dph}, 20/35 \text{ dph}, 20/50 \text{ dph}$ and $18/\text{adults}$). **i.** Average instantaneous firing frequency as a function of current injected for each age group (two-way ANOVA with Tukey's post hoc; $P = 0.004$, $F(15, 361) = 2.320$, $N(\text{cells/age}) = 18/20 \text{ dph}, 20/35 \text{ dph}, 20/50 \text{ dph}$ and $18/\text{adults}$). **j.** Fold-change of the maximum depolarization rate from the 1st to the 2nd AP as a function of current injected for each age group (two-way ANOVA with Tukey's post hoc; $P < 0.0001$, $F(15, 348) = 6.774$, $N(\text{cells/age}) = 18/20 \text{ dph}, 20/35 \text{ dph}, 20/50 \text{ dph}$ and $18/\text{adults}$).

Figure 7. Effects of Navβ4 C-terminal peptide (β4-WT) on I_{NaR} and intrinsic excitability in RAPNs of 20 dph males. **a1.** **a2.** Representative traces of I_{NaT} elicited at +30 mV followed by I_{NaR} elicited at -30 mV immediately after (black) and 7 min after (red) achieving whole-cell configuration with β4-WT (KKLITFILKKTREK) in the patch pipette. The regions in the red dashed boxes are enlarged in the insets. **b.** Time-dependent increase in the normalized I_{NaR} to I_{NaT} ratio caused by β4-WT, measured at the -30 mV test potential in individual RAPNs (mean ± SEM in black); paired t-test, $N=10$ and 9 RAPNs for β4-WT and β4-Scr, respectively. **c.** Representative traces of I_{NaT} elicited at +30 mV followed by I_{NaR} elicited at -30 mV immediately after (black) and 7 min after (blue) achieving whole-cell configuration with β4-Scr (KIKIRFKTKTLELK) in the patch pipette. The region in the red dashed box is enlarged in the inset. **d.** Lack of time-dependent increase in normalized I_{NaR} to I_{NaT} ratio by β4-Scr, measured at the -30 mV test potential in individual RAPNs (mean ± SEM in black); paired t-test, $N=10$ and 9 RAPNs for β4-WT and β4-Scr, respectively. **e.** Fold-changes in normalized I_{NaR} to I_{NaT} ratio between 0 and 7 min for RAPNs exposed to β4-WT or β4-Scr in the patch pipette; Student's t-test; $N=10$ and 9 RAPNs for β4-WT and β4-Scr, respectively. **f.** Representative traces of the first APs elicited by a +300 pA current injection at four time points after entering the whole-cell configuration with β4-WT in the patch pipette; the APs are aligned at their peak. **g.** Phase plane plots from the first elicited AP recorded immediately (black) or 15 min (red) after entering the whole-cell configuration in cells exposed to β4-WT. The blue arrows point to the spikelet and resulting alteration in the repolarization rate in the phase plane plot. **h.** Time-dependent effects of β4-WT on the 1st AP half-width in individual cells (mean ± SEM in black; paired t-test). **i.** Representative traces of the first APs elicited by a +300 pA current injection at four time points after entering the whole-cell configuration with β4-Scr in the patch pipette; the APs are aligned at their peak. **j.** Phase plane plots from the first elicited AP recorded immediately (black) or 15 min (red) after entering the whole-cell configuration for cells perfused with the β4-Scr. **k.** Time-dependent effects of β4-Scr on the 1st AP half-width in individual cells (mean ± SEM in black; paired t-test). $N=12$ and 15 cells recorded with β4-WT and β4-Scr respectively.

Figure 8. Dynamic clamping of spiking RAPNs in juvenile and adult male finches. **a.** Family of recorded I_{NaR} (same as in Fig. 2C). **b.** Family of modeled I_{NaR} currents (same voltage protocol that elicited currents in Fig. 1c; same scale as in a). **c.** Overlay of the current-voltage relationship of peak I_{NaR} comparing the modeled (red) and average recorded values (black; same as in Fig. 2e). **d.** Overlay of the time constant of decay (τ) as a function of voltage in the modeled (red) and recorded currents (black). **e.** APs elicited in 20 dph RAPNs before (OFF; black) or during dynamic clamp-mediated addition (ON; red) of the *in silico* I_{NaR} (blue) peaking during the AP repolarization phase. APs were triggered by a +300 pA current injection. **f.** Paired data of the instantaneous firing frequency change recorded in 20 dph RAPNs upon dynamic clamp-mediated addition (ON; pink) compared to the control (OFF; grey) condition as a function of conductance (mean $\pm$ SEM in black, paired t-test). **g.** APs elicited in adult RAPNs before (OFF; black) or during dynamic clamp-mediated subtraction (ON; red) of the *in silico* I_{NaR} (blue) peaking during the AP repolarization phase. APs were triggered as in (e). **h.** Paired data of the instantaneous firing frequency change recorded in adult RAPNs upon dynamic clamp-mediated subtraction (ON; pink) compared to the control (OFF; grey) condition as a function of conductance (mean $\pm$ SEM in black, paired t-test). N = 8 20 dph and 5 adult RAPNs; In F & G: * = $P \leq 0.05$, ** = $P \leq 0.01$.

Figure 9. Analysis of Navβ4 mRNA and intrinsic excitable properties in RA of female zebra finches across ages. **a.** Representative *in situ* hybridization images for Navβ4 across ages; black arrowheads indicate RA borders. **b.** Views of adjacent sections to those in (a), stained for Nissl; black arrowheads indicate RA borders. **c.** Comparison of Navβ4 expression levels (normalized optical density) across age groups within RA (one-way ANOVA with Tukey's post hoc; $P = 0.06$, 3.290 (2, 10), N (birds/age) = 5/20 dph, 4/35 dph and 4/50 dph females). **d.** Comparison of the proportions of Navβ4-expressing cells across age groups within RA (one-way ANOVA with Tukey's post hoc; $P = 0.0001$, F (2, 10) = 24.95; N (birds/age) = 5/20 dph, 4/35 dph and 4/50 dph females; stars depict significant age differences in RA determined by post hoc analyses; $*$ = $P \leq 0.01$). **e.** Representative currents from RAPNs at each age group during the -30 mV test potential shown at the top. The large I_{NaT} peaks have been truncated. **f.** Average I-V curves for the peak I_{NaR} in RA neurons at each age group; no significant groups differences seen (two-way ANOVA with Tukey's post hoc; $P = 0.83$, F (12, 140) = 0.6103, N (cells/age) = 9/20 dph, 10/35 dph and 4/50 dph females). Grey dashed line shows I-V relationship for 50 dph male (same as in Fig. 4c). **g.** I-V curves after normalization of the peak I_{NaR} to the peak I_{NaT} measured in a given sweep then averaged across cells (two-way ANOVA with Tukey's post hoc; $P = 0.48$, F (12, 140) = 0.9678, N (cells/age) = 9/20 dph, 10/35 dph and 4/50 dph females). Grey dashed line shows I-V relationship for 50 dph male (same as in Fig. 4d).

Figure 10. Age-dependent changes in intrinsic excitable properties of RAPNs in female and male zebra finches. **a-c.** Left: Representative AP trains elicited by a 500 pA current injection in RAPNs in males (First 10 APs; black; same as in Fig. 6d-f, left) and females (red) across ages. Right: APs #1, 2 and 16-21 from the traces on the left aligned at the peaks for each age group in females. **d-f.** Phase plane plots from APs #1, 2, and 16-21 plotted at the same scale for all age groups for females (left) and males (right; same as in Fig. 6d-f, right). **g-i.** 3D dot plots showing the instantaneous firing frequency, number of spikes per second and the fold-change in maximum depolarization rate between the 2nd and 1st APs during a 1 sec 500 pA current injection in male (black) and female (red) RAPNs. N (cells/age) = 21/20 dph, 23/35 dph and 7/50 dph females. Refer to Fig. 6 for male data.

References:

- 1 Ebbesen, C. L. & Brecht, M. Motor cortex - to act or not to act? *Nat Rev Neurosci* **18**, 694-705, doi:10.1038/nrn.2017.119 (2017).
- 2 Suter, B. A., Migliore, M. & Shepherd, G. M. Intrinsic electrophysiology of mouse corticospinal neurons: a class-specific triad of spike-related properties. *Cereb Cortex* **23**, 1965-1977, doi:10.1093/cercor/bhs184 (2013).
- 3 Oswald, M. J., Tantirigama, M. L., Sonntag, I., Hughes, S. M. & Empson, R. M. Diversity of layer 5 projection neurons in the mouse motor cortex. *Front Cell Neurosci* **7**, 174, doi:10.3389/fncel.2013.00174 (2013).
- 4 Paz, J. T. *et al.* Multiple forms of activity-dependent intrinsic plasticity in layer V cortical neurones in vivo. *J Physiol* **587**, 3189-3205, doi:10.1113/jphysiol.2009.169334 (2009).
- 5 Delvendahl, I., Jung, N. H., Kuhnke, N. G., Ziemann, U. & Mall, V. Plasticity of motor threshold and motor-evoked potential amplitude--a model of intrinsic and synaptic plasticity in human motor cortex? *Brain Stimul* **5**, 586-593, doi:10.1016/j.brs.2011.11.005 (2012).
- 6 Shim, H. G., Lee, Y. S. & Kim, S. J. The Emerging Concept of Intrinsic Plasticity: Activity-dependent Modulation of Intrinsic Excitability in Cerebellar Purkinje Cells and Motor Learning. *Exp Neurobiol* **27**, 139-154, doi:10.5607/en.2018.27.3.139 (2018).
- 7 Eshra, A., Hirrlinger, P. & Hallermann, S. Enriched Environment Shortens the Duration of Action Potentials in Cerebellar Granule Cells. *Front Cell Neurosci* **13**, 289, doi:10.3389/fncel.2019.00289 (2019).
- 8 Nakajima, K., Maier, M. A., Kirkwood, P. A. & Lemon, R. N. Striking differences in transmission of corticospinal excitation to upper limb motoneurons in two primate species. *J Neurophysiol* **84**, 698-709, doi:10.1152/jn.2000.84.2.698 (2000).
- 9 Vigneswaran, G., Kraskov, A. & Lemon, R. N. Large identified pyramidal cells in macaque motor and premotor cortex exhibit "thin spikes": implications for cell type classification. *J Neurosci* **31**, 14235-14242, doi:10.1523/JNEUROSCI.3142-11.2011 (2011).
- 10 Soares, D. *et al.* Expression of Kv3.1b potassium channel is widespread in macaque motor cortex pyramidal cells: A histological comparison between rat and macaque. *J Comp Neurol* **525**, 2164-2174, doi:10.1002/cne.24192 (2017).
- 11 Chen, W., Zhang, J. J., Hu, G. Y. & Wu, C. P. Electrophysiological and morphological properties of pyramidal and nonpyramidal neurons in the cat motor cortex in vitro. *Neuroscience* **73**, 39-55, doi:10.1016/0306-4522(96)00009-7 (1996).
- 12 Brainard, M. S. & Doupe, A. J. What songbirds teach us about learning. *Nature* **417**, 351-358, doi:10.1038/417351a (2002).
- 13 Gunturkun, O. & Bugnyar, T. Cognition without Cortex. *Trends Cogn Sci* **20**, 291-303, doi:10.1016/j.tics.2016.02.001 (2016).
- 14 Clayton, N. S. & Emery, N. J. Avian Models for Human Cognitive Neuroscience: A Proposal. *Neuron* **86**, 1330-1342, doi:10.1016/j.neuron.2015.04.024 (2015).
- 15 Calabrese, A. & Woolley, S. M. Coding principles of the canonical cortical microcircuit in the avian brain. *Proc Natl Acad Sci U S A* **112**, 3517-3522, doi:10.1073/pnas.1408545112 (2015).
- 16 Dugas-Ford, J., Rowell, J. J. & Ragsdale, C. W. Cell-type homologies and the origins of the neocortex. *Proc Natl Acad Sci U S A* **109**, 16974-16979, doi:10.1073/pnas.1204773109 (2012).
- 17 Karten, H. J. Vertebrate brains and evolutionary connectomics: on the origins of the mammalian 'neocortex'. *Philos Trans R Soc Lond B Biol Sci* **370**, doi:10.1098/rstb.2015.0060 (2015).
- 18 Stacho, M. *et al.* A cortex-like canonical circuit in the avian forebrain. *Science* **369**, doi:10.1126/science.abc5534 (2020).

1020 19 Pfenning, A. R. *et al.* Convergent transcriptional specializations in the brains of humans and
1021 song-learning birds. *Science* **346**, 1256846, doi:10.1126/science.1256846 (2014).
1022 20 Nottebohm, F., Stokes, T. M. & Leonard, C. M. Central control of song in the canary, *Serinus*
1023 *canarius*. *J Comp Neurol* **165**, 457-486, doi:10.1002/cne.901650405 (1976).
1024 21 Gurney, M. E. Hormonal control of cell form and number in the zebra finch song system. *J*
1025 *Neurosci* **1**, 658-673 (1981).
1026 22 Paton, J. A., Manogue, K. R. & Nottebohm, F. Bilateral organization of the vocal control pathway
1027 in the budgerigar, *Melopsittacus undulatus*. *Journal of Neuroscience* **1**, 1279-1288. (1981).
1028 23 Schmidt, M. F. & Martin Wild, J. The respiratory-vocal system of songbirds: anatomy, physiology,
1029 and neural control. *Prog Brain Res* **212**, 297-335, doi:10.1016/B978-0-444-63488-7.00015-X
1030 (2014).
1031 24 Spiro, J. E., Dalva, M. B. & Mooney, R. Long-range inhibition within the zebra finch song nucleus
1032 RA can coordinate the firing of multiple projection neurons. *J Neurophysiol* **81**, 3007-3020,
1033 doi:10.1152/jn.1999.81.6.3007 (1999).
1034 25 Mao, B. Q., Hamzei-Sichani, F., Aronov, D., Froemke, R. C. & Yuste, R. Dynamics of spontaneous
1035 activity in neocortical slices. *Neuron* **32**, 883-898, doi:10.1016/s0896-6273(01)00518-9 (2001).
1036 26 Zhang, Z. W. Maturation of layer V pyramidal neurons in the rat prefrontal cortex: intrinsic
1037 properties and synaptic function. *J Neurophysiol* **91**, 1171-1182, doi:10.1152/jn.00855.2003
1038 (2004).
1039 27 Beaulieu-Laroche, L. *et al.* Enhanced Dendritic Compartmentalization in Human Cortical
1040 Neurons. *Cell* **175**, 643-651 e614, doi:10.1016/j.cell.2018.08.045 (2018).
1041 28 Elemans, C. P., Mead, A. F., Rome, L. C. & Goller, F. Superfast vocal muscles control song
1042 production in songbirds. *PLoS One* **3**, e2581, doi:10.1371/journal.pone.0002581 (2008).
1043 29 Goller, F. & Suthers, R. A. Role of syringeal muscles in gating airflow and sound production in
1044 singing brown thrashers. *J Neurophysiol* **75**, 867-876, doi:10.1152/jn.1996.75.2.867 (1996).
1045 30 Vicario, D. S. Contributions of syringeal muscles to respiration and vocalization in the zebra
1046 finch. *J Neurobiol* **22**, 63-73, doi:10.1002/neu.480220107 (1991).
1047 31 Chi, Z. & Margoliash, D. Temporal precision and temporal drift in brain and behavior of zebra
1048 finch song. *Neuron* **32**, 899-910 (2001).
1049 32 Olveczky, B. P., Otchy, T. M., Goldberg, J. H., Aronov, D. & Fee, M. S. Changes in the neural
1050 control of a complex motor sequence during learning. *J Neurophysiol* **106**, 386-397,
1051 doi:10.1152/jn.00018.2011 (2011).
1052 33 Adam, I. & Elemans, C. P. H. Increasing muscle speed drives changes in the neuromuscular
1053 transform of motor commands during postnatal development in songbirds. *J Neurosci*,
1054 doi:10.1523/JNEUROSCI.0111-20.2020 (2020).
1055 34 Adret, P. & Margoliash, D. Metabolic and neural activity in the song system nucleus robustus
1056 archistriatalis: effect of age and gender. *J Comp Neurol* **454**, 409-423, doi:10.1002/cne.10459
1057 (2002).
1058 35 Gallant, J. R. & O'Connell, L. A. Studying convergent evolution to relate genotype to behavioral
1059 phenotype. *J Exp Biol* **223**, doi:10.1242/jeb.213447 (2020).
1060 36 Zakon, H. H., Lu, Y., Zwickl, D. J. & Hillis, D. M. Sodium channel genes and the evolution of
1061 diversity in communication signals of electric fishes: convergent molecular evolution. *Proc Natl*
1062 *Acad Sci U S A* **103**, 3675-3680, doi:10.1073/pnas.0600160103 (2006).
1063 37 McAnelly, M. L. & Zakon, H. H. Coregulation of voltage-dependent kinetics of Na(+) and K(+)
1064 currents in electric organ. *J Neurosci* **20**, 3408-3414 (2000).
1065 38 Markham, M. R., Kaczmarek, L. K. & Zakon, H. H. A sodium-activated potassium channel
1066 supports high-frequency firing and reduces energetic costs during rapid modulations of action
1067 potential amplitude. *J Neurophysiol* **109**, 1713-1723, doi:10.1152/jn.00875.2012 (2013).

1068 39 Miller, M. N., Cheung, C. Y. J. & Brainard, M. S. Vocal learning promotes patterned inhibitory
1069 connectivity. *Nat Commun* **8**, 2105, doi:10.1038/s41467-017-01914-5 (2017).

1070 40 Liao, S. Q., Hou, G. Q., Liu, X. L., Long, C. & Li, D. F. Electrophysiological properties of neurons in
1071 the robust nucleus of the arcopallium of adult male zebra finches. *Neurosci Lett* **487**, 234-239,
1072 doi:10.1016/j.neulet.2010.10.029 (2011).

1073 41 Garst-Orozco, J., Babadi, B. & Olveczky, B. P. A neural circuit mechanism for regulating vocal
1074 variability during song learning in zebra finches. *Elife* **3**, e03697, doi:10.7554/eLife.03697 (2014).

1075 42 Kittelberger, J. M. & Mooney, R. Lesions of an avian forebrain nucleus that disrupt song
1076 development alter synaptic connectivity and transmission in the vocal premotor pathway. *J*
1077 *Neurosci* **19**, 9385-9398 (1999).

1078 43 McCormick, D. A., Shu, Y. & Yu, Y. Neurophysiology: Hodgkin and Huxley model--still standing?
1079 *Nature* **445**, E1-2; discussion E2-3, doi:10.1038/nature05523 (2007).

1080 44 Aronov, D. & Fee, M. S. Natural changes in brain temperature underlie variations in song tempo
1081 during a mating behavior. *PLoS One* **7**, e47856, doi:10.1371/journal.pone.0047856 (2012).

1082 45 Long, M. A. & Fee, M. S. Using temperature to analyse temporal dynamics in the songbird motor
1083 pathway. *Nature* **456**, 189-194, doi:10.1038/nature07448 (2008).

1084 46 Yu, Y., Hill, A. P. & McCormick, D. A. Warm body temperature facilitates energy efficient cortical
1085 action potentials. *PLoS Comput Biol* **8**, e1002456, doi:10.1371/journal.pcbi.1002456 (2012).

1086 47 McCormick, D. A., Connors, B. W., Lighthall, J. W. & Prince, D. A. Comparative electrophysiology
1087 of pyramidal and sparsely spiny stellate neurons of the neocortex. *J Neurophysiol* **54**, 782-806,
1088 doi:10.1152/jn.1985.54.4.782 (1985).

1089 48 Hedrick, T. & Waters, J. Effect of temperature on spiking patterns of neocortical layer 2/3 and
1090 layer 6 pyramidal neurons. *Front Neural Circuits* **6**, 28, doi:10.3389/fncir.2012.00028 (2012).

1091 49 Friedrich, S. R., Lovell, P. V., Kaser, T. M. & Mello, C. V. Exploring the molecular basis of neuronal
1092 excitability in a vocal learner. *BMC Genomics* **20**, 629, doi:10.1186/s12864-019-5871-2 (2019).

1093 50 Raman, I. M. & Bean, B. P. Resurgent sodium current and action potential formation in
1094 dissociated cerebellar Purkinje neurons. *J Neurosci* **17**, 4517-4526 (1997).

1095 51 Grieco, T. M., Malhotra, J. D., Chen, C., Isom, L. L. & Raman, I. M. Open-channel block by the
1096 cytoplasmic tail of sodium channel $\beta 4$ as a mechanism for resurgent sodium current. *Neuron* **45**,
1097 233-244 (2005).

1098 52 Lewis, A. H. & Raman, I. M. Resurgent current of voltage-gated Na(+) channels. *J Physiol* **592**,
1099 4825-4838, doi:10.1113/jphysiol.2014.277582 (2014).

1100 53 Lewis, A. H. & Raman, I. M. Cross-species conservation of open-channel block by Na channel
1101 beta4 peptides reveals structural features required for resurgent Na current. *J Neurosci* **31**,
1102 11527-11536, doi:10.1523/JNEUROSCI.1428-11.2011 (2011).

1103 54 Hong, H., Lu, T., Wang, X., Wang, Y. & Sanchez, J. T. Resurgent sodium current promotes action
1104 potential firing in the avian auditory brainstem. *J Physiol* **596**, 423-443, doi:10.1113/JP275083
1105 (2018).

1106 55 Gobes, S. M. H., Jennings, R. B. & Maeda, R. K. The sensitive period for auditory-vocal learning in
1107 the zebra finch: Consequences of limited-model availability and multiple-tutor paradigms on
1108 song imitation. *Behav Processes* **163**, 5-12, doi:10.1016/j.beproc.2017.07.007 (2019).

1109 56 Konishi, M. & Akutagawa, E. Neuronal growth, atrophy and death in a sexually dimorphic song
1110 nucleus in the zebra finch brain. *Nature* **315**, 145-147, doi:10.1038/315145a0 (1985).

1111 57 Nixdorf-Bergweiler, B. E. Divergent and parallel development in volume sizes of telencephalic
1112 song nuclei in male and female zebra finches. *J Comp Neurol* **375**, 445-456,
1113 doi:10.1002/(SICI)1096-9861(19961118)375:3<445::AID-CNE7>3.0.CO;2-2 (1996).

1114 58 Bottjer, S. W., Halsema, K. A., Brown, S. A. & Miesner, E. A. in *Journal of Comparative Neurology*
1115 Vol. 279 312-326 (Alan R. Liss, Inc., 1989).

1116 59 Stark, L. L. & Perkel, D. J. Two-stage, input-specific synaptic maturation in a nucleus essential for
1117 vocal production in the zebra finch. *J Neurosci* **19**, 9107-9116 (1999).

1118 60 Vicario, D. S. Organization of the zebra finch song control system: II. Functional organization of
1119 outputs from nucleus Robustus archistriatalis. *J Comp Neurol* **309**, 486-494,
1120 doi:10.1002/cne.903090405 (1991).

1121 61 White, S. A., Livingston, F. S. & Mooney, R. Androgens modulate NMDA receptor-mediated
1122 EPSCs in the zebra finch song system. *J Neurophysiol* **82**, 2221-2234 (1999).

1123 62 Eales, L. A. Song learning in zebra finches: some effects of song model availability on what is
1124 learnt and when. *Animal Behavior* **33**, 1293-1300 (1985).

1125 63 Safronov, B. V., Wolff, M. & Vogel, W. Functional distribution of three types of Na⁺ channel on
1126 soma and processes of dorsal horn neurones of rat spinal cord. *J Physiol* **503 (Pt 2)**, 371-385,
1127 doi:10.1111/j.1469-7793.1997.371bh.x (1997).

1128 64 Edmonds, B. W., Gregory, F. D. & Schweizer, F. E. Evidence that fast exocytosis can be
1129 predominantly mediated by vesicles not docked at active zones in frog saccular hair cells. *J*
1130 *Physiol* **560**, 439-450, doi:10.1113/jphysiol.2004.066035 (2004).

1131 65 Almog, M., Barkai, T., Lampert, A. & Kornegreen, A. Voltage-Gated Sodium Channels in
1132 Neocortical Pyramidal Neurons Display Cole-Moore Activation Kinetics. *Front Cell Neurosci* **12**,
1133 187, doi:10.3389/fncel.2018.00187 (2018).

1134 66 Magistretti, J., Castelli, L., Forti, L. & D'Angelo, E. Kinetic and functional analysis of transient,
1135 persistent and resurgent sodium currents in rat cerebellar granule cells in situ: an
1136 electrophysiological and modelling study. *J Physiol* **573**, 83-106,
1137 doi:10.1113/jphysiol.2006.106682 (2006).

1138 67 Kim, J. H., Kushmerick, C. & Von Gersdorff, H. Presynaptic resurgent Na⁺ currents sculpt the
1139 action potential waveform and increase firing reliability at a CNS nerve terminal. *J Neurosci* **30**,
1140 15479-15490 (2010).

1141 68 Champoux, K. L., Miller, K. E. & Perkel, D. J. Differential development of myelin in zebra finch
1142 song nuclei. *J Comp Neurol*, doi:10.1002/cne.25019 (2020).

1143 69 Vaaga, C. E., Brown, S. T. & Raman, I. M. Cerebellar modulation of synaptic input to freezing-
1144 related neurons in the periaqueductal gray. *Elife* **9**, doi:10.7554/eLife.54302 (2020).

1145 70 O'Leary, T. & Marder, E. Temperature-Robust Neural Function from Activity-Dependent Ion
1146 Channel Regulation. *Curr Biol* **26**, 2935-2941, doi:10.1016/j.cub.2016.08.061 (2016).

1147 71 Botta, P., de Souza, F. M., Sangrey, T., De Schutter, E. & Valenzuela, C. F. Alcohol excites
1148 cerebellar Golgi cells by inhibiting the Na⁺/K⁺ ATPase. *Neuropsychopharmacology* **35**, 1984-
1149 1996, doi:10.1038/npp.2010.76 (2010).

1150 72 Lacey, M. G. *et al.* Spike firing and IPSPs in layer V pyramidal neurons during beta oscillations in
1151 rat primary motor cortex (M1) in vitro. *PLoS One* **9**, e85109, doi:10.1371/journal.pone.0085109
1152 (2014).

1153 73 Tsubo, Y., Takada, M., Reyes, A. D. & Fukai, T. Layer and frequency dependencies of phase
1154 response properties of pyramidal neurons in rat motor cortex. *Eur J Neurosci* **25**, 3429-3441,
1155 doi:10.1111/j.1460-9568.2007.05579.x (2007).

1156 74 Palmer, L. M. & Stuart, G. J. Site of action potential initiation in layer 5 pyramidal neurons. *J*
1157 *Neurosci* **26**, 1854-1863, doi:10.1523/JNEUROSCI.4812-05.2006 (2006).

1158 75 Eyal, G., Mansvelder, H. D., de Kock, C. P. & Segev, I. Dendrites impact the encoding capabilities
1159 of the axon. *J Neurosci* **34**, 8063-8071, doi:10.1523/JNEUROSCI.5431-13.2014 (2014).

1160 76 Lazarov, E. *et al.* An axon initial segment is required for temporal precision in action potential
1161 encoding by neuronal populations. *Sci Adv* **4**, eaau8621, doi:10.1126/sciadv.aau8621 (2018).

1162 77 Testa-Silva, G. *et al.* High bandwidth synaptic communication and frequency tracking in human
1163 neocortex. *PLoS Biol* **12**, e1002007, doi:10.1371/journal.pbio.1002007 (2014).

1164 78 Lovell, P. V., Carleton, J. B. & Mello, C. V. Genomics analysis of potassium channel genes in
1165 songbirds reveals molecular specializations of brain circuits for the maintenance and production
1166 of learned vocalizations. *BMC Genomics* **14**, 470, doi:10.1186/1471-2164-14-470 (2013).
1167 79 Kaczmarek, L. K. & Zhang, Y. Kv3 Channels: Enablers of Rapid Firing, Neurotransmitter Release,
1168 and Neuronal Endurance. *Physiol Rev* **97**, 1431-1468, doi:10.1152/physrev.00002.2017 (2017).
1169 80 Schlosser, B., ten Bruggencate, G. & Sutor, B. The intracellular tracer Neurobiotin alters
1170 electrophysiological properties of rat neostriatal neurons. *Neurosci Lett* **249**, 13-16,
1171 doi:10.1016/s0304-3940(98)00366-8 (1998).
1172 81 Mooney, R. Synaptic basis for developmental plasticity in a birdsong nucleus. *J Neurosci* **12**,
1173 2464-2477 (1992).
1174 82 Daou, A. & Margoliash, D. Intrinsic neuronal properties represent song and error in zebra finch
1175 vocal learning. *Nat Commun* **11**, 952, doi:10.1038/s41467-020-14738-7 (2020).
1176 83 Hallermann, S., de Kock, C. P., Stuart, G. J. & Kole, M. H. State and location dependence of action
1177 potential metabolic cost in cortical pyramidal neurons. *Nat Neurosci* **15**, 1007-1014,
1178 doi:10.1038/nn.3132 (2012).
1179 84 Alle, H., Roth, A. & Geiger, J. R. Energy-efficient action potentials in hippocampal mossy fibers.
1180 *Science* **325**, 1405-1408, doi:10.1126/science.1174331 (2009).
1181 85 Fohlmeister, J. F. A nerve model of greatly increased energy-efficiency and encoding flexibility
1182 over the Hodgkin-Huxley model. *Brain Res* **1296**, 225-233, doi:10.1016/j.brainres.2009.06.101
1183 (2009).
1184 86 Hasenstaub, A., Otte, S., Callaway, E. & Sejnowski, T. J. Metabolic cost as a unifying principle
1185 governing neuronal biophysics. *Proc Natl Acad Sci U S A* **107**, 12329-12334,
1186 doi:10.1073/pnas.0914886107 (2010).
1187 87 Sengupta, B., Stemmler, M., Laughlin, S. B. & Niven, J. E. Action potential energy efficiency varies
1188 among neuron types in vertebrates and invertebrates. *PLoS Comput Biol* **6**, e1000840,
1189 doi:10.1371/journal.pcbi.1000840 (2010).
1190 88 Aronov, D., Andalman, A. S. & Fee, M. S. A specialized forebrain circuit for vocal babbling in the
1191 juvenile songbird. *Science* **320**, 630-634, doi:10.1126/science.1155140 (2008).
1192 89 Madeja, M. Do neurons have a reserve of sodium channels for the generation of action
1193 potentials? A study on acutely isolated CA1 neurons from the guinea-pig hippocampus. *Eur J*
1194 *Neurosci* **12**, 1-7, doi:10.1046/j.1460-9568.2000.00871.x (2000).
1195 90 Zhao, J., O'Leary, M. E. & Chahine, M. Regulation of Nav1.6 and Nav1.8 peripheral nerve Na⁺
1196 channels by auxiliary beta-subunits. *J Neurophysiol* **106**, 608-619, doi:10.1152/jn.00107.2011
1197 (2011).
1198 91 Hull, J. M. & Isom, L. L. Voltage-gated sodium channel beta subunits: The power outside the
1199 pore in brain development and disease. *Neuropharmacology* **132**, 43-57,
1200 doi:10.1016/j.neuropharm.2017.09.018 (2018).
1201 92 Wimmer, V. C. *et al.* Sodium channel beta1 subunit localizes to axon initial segments of
1202 excitatory and inhibitory neurons and shows regional heterogeneity in mouse brain. *J Comp*
1203 *Neurol* **523**, 814-830, doi:10.1002/cne.23715 (2015).
1204 93 Brackenbury, W. J. *et al.* Functional reciprocity between Na⁺ channel Nav1. 6 and β 1 subunits in
1205 the coordinated regulation of excitability and neurite outgrowth. *PNAS* **107**, 2283-2288 (2010).
1206 94 Van Wart, A. & Matthews, G. Impaired firing and cell-specific compensation in neurons lacking
1207 nav1.6 sodium channels. *J Neurosci* **26**, 7172-7180, doi:10.1523/JNEUROSCI.1101-06.2006
1208 (2006).
1209 95 Smith, M. R., Smith, R. D., Plummer, N. W., Meisler, M. H. & Goldin, A. L. Functional analysis of
1210 the mouse Scn8a sodium channel. *J Neurosci* **18**, 6093-6102 (1998).

1211 96 Barbosa, C. *et al.* Navbeta4 regulates fast resurgent sodium currents and excitability in sensory
1212 neurons. *Mol Pain* **11**, 60, doi:10.1186/s12990-015-0063-9 (2015).

1213 97 Bant, J. S. & Raman, I. M. Control of transient, resurgent, and persistent current by open-
1214 channel block by Na channel beta4 in cultured cerebellar granule neurons. *Proc Natl Acad Sci U S A* **107**, 12357-12362, doi:10.1073/pnas.1005633107 (2010).

1215 98 Ransdell, J. L. *et al.* Loss of Navbeta4-Mediated Regulation of Sodium Currents in Adult Purkinje
1216 Neurons Disrupts Firing and Impairs Motor Coordination and Balance. *Cell Rep* **20**, 1502,
1217 doi:10.1016/j.celrep.2017.07.072 (2017).

1218 99 White, H. V., Brown, S. T., Bozza, T. C. & Raman, I. M. Effects of FGF14 and Navbeta4 deletion on
1219 transient and resurgent Na current in cerebellar Purkinje neurons. *J Gen Physiol* **151**, 1300-1318,
1220 doi:10.1085/jgp.201912390 (2019).

1221 100 Leao, R. M. *et al.* Presynaptic Na⁺ channels: locus, development, and recovery from inactivation
1222 at a high-fidelity synapse. *J Neurosci* **25**, 3724-3738, doi:10.1523/JNEUROSCI.3983-04.2005
1223 (2005).

1224 101 Kim, J. H., Renden, R. & von Gersdorff, H. Dysmyelination of auditory afferent axons increases
1225 the jitter of action potential timing during high-frequency firing. *J Neurosci* **33**, 9402-9407,
1226 doi:10.1523/JNEUROSCI.3389-12.2013 (2013).

1227 102 Lein, E. S. *et al.* Genome-wide atlas of gene expression in the adult mouse brain. *Nature* **445**,
1228 168-176, doi:10.1038/nature05453 (2007).

1229 103 Buffington, S. A. & Rasband, M. N. Na⁺ channel-dependent recruitment of Navbeta4 to axon
1230 initial segments and nodes of Ranvier. *J Neurosci* **33**, 6191-6202, doi:10.1523/JNEUROSCI.4051-
1231 12.2013 (2013).

1232 104 Wang, S. *et al.* Sex differences of excitatory synaptic transmission in RA projection neurons of
1233 adult zebra finches. *Neurosci Lett* **582**, 75-80, doi:10.1016/j.neulet.2014.09.001 (2014).

1234 105 George, J. M. *et al.* Acute social isolation alters neurogenomic state in songbird forebrain. *Proc*
1235 *Natl Acad Sci U S A*, doi:10.1073/pnas.1820841116 (2019).

1236 106 Carleton, J. B. *et al.* An optimized protocol for high-throughput in situ hybridization of zebra
1237 finch brain. *Cold Spring Harb Protoc* **2014**, 1249-1258, doi:10.1101/pdb.prot084582 (2014).

1238 107 Replogle, K. *et al.* The Songbird Neurogenomics (SoNG) Initiative: community-based tools and
1239 strategies for study of brain gene function and evolution. *BMC Genomics* **9**, 131,
1240 doi:10.1186/1471-2164-9-131 (2008).

1241 108 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**,
1242 676-682, doi:10.1038/nmeth.2019 (2012).

1243 109 Alexander, R. P. D., Mitry, J., Sareen, V., Khadra, A. & Bowie, D. Cerebellar Stellate Cell
1244 Excitability Is Coordinated by Shifts in the Gating Behavior of Voltage-Gated Na(+) and A-Type
1245 K(+) Channels. *eNeuro* **6**, doi:10.1523/ENEURO.0126-19.2019 (2019).

1246 110 Dagostin, A. A., Lovell, P. V., Hilscher, M. M., Mello, C. V. & Leao, R. M. Control of Phasic Firing
1247 by a Background Leak Current in Avian Forebrain Auditory Neurons. *Front Cell Neurosci* **9**, 471,
1248 doi:10.3389/fncel.2015.00471 (2015).

1249 111 McDougal, R. A. *et al.* Twenty years of ModelDB and beyond: building essential modeling tools
1250 for the future of neuroscience. *J Comput Neurosci* **42**, 1-10, doi:10.1007/s10827-016-0623-7
1251 (2017).

1252 112 Ceballos, C. C., Roque, A. C. & Leao, R. M. A Negative Slope Conductance of the Persistent
1253 Sodium Current Prolongs Subthreshold Depolarizations. *Biophys J* **113**, 2207-2217,
1254 doi:10.1016/j.bpj.2017.06.047 (2017).

1255 113 Lovell, P. V. *et al.* ZEBRA: Zebra finch Expression Brain Atlas-A resource for comparative
1256 molecular neuroanatomy and brain evolution studies. *J Comp Neurol* **528**, 2099-2131,
1257 doi:10.1002/cne.24879 (2020).

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Author Contributions:

B.M.Z, P.V.L, C.V.M and H.v.G designed the research. B.M.Z, A.A.N., A.D. performed the research, B.M.Z., A.A.N., A.D., P.V.L, C.V.M. and H.v.G. analyzed the data, B.M.Z, P.V.L, C.V.M and H.v.G wrote the paper.

Competing Interests:

None