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Title

Unravelling the molecular players at the cholinergic efferent synapse of the zebrafish lateral line

Abbreviated title

Nicotinic receptor at the lateral line efferent synapse

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Contributions ACF, MM, PVP and ABE designed research, ACF, MM, TC, LS and CW performed research, IM, SG, SD and JCG contributed unpublished reagents/analytic tools, ACF and PVP analyzed data, ACF, PVP and ABE wrote the paper.

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Abstract

The lateral line (LL) is a sensory system that allows fish and amphibians to detect water currents. LL responsiveness is modulated by efferent neurons which aid to distinguish between external and self-generated stimuli, maintaining sensitivity to relevant cues. One component of the efferent system is cholinergic, the activation of which inhibits afferent activity. LL hair cells (HC) share structural, functional and molecular similarities with those of the cochlea, making them a popular model for studying human hearing and balance disorders. Due to these commonalities, one could propose that the receptor at the LL efferent synapse is a α9α10 nicotinic cholinergic one (nAChR). However, the identities of the molecular players underlying acetylcholine (ACh)-mediated inhibition in the LL remain unknown. Surprisingly, through the analysis of single-cell expression studies and *in situ* hybridization, we describe that α9, but not α10 subunits, are enriched in zebrafish HC. Moreover, the heterologous expression of zebrafish α9 subunits indicates that homomeric receptors are functional and exhibit robust ACh-gated currents blocked by α-Bungarotoxin and strychnine. In addition, *in vivo* Ca^{2+} imaging on mechanically-stimulated zebrafish LL HC show that ACh elicits a decrease in evoked Ca^{2+} signals, irrespective of HC polarity. This effect is blocked by both α-Bungarotoxin and apamin, indicating coupling of ACh-mediated effects to SK potassium channels. Our results indicate that an α9-containing (α9*) nAChR operates at the zebrafish LL efferent synapse. Moreover, the activation of α9* nAChRs most likely leads to LL HC hyperpolarization served by the activation of Ca^{2+}-dependent SK potassium channels.

Significance Statement

The fish lateral line (LL) mechanosensory system shares structural, functional and molecular similarities with those of the mammalian cochlea. Thus, it has become an accessible model for studying human hearing and balance disorders. However, the molecular players serving efferent control of LL hair cell (HC) activity have not been identified. Here we demonstrate that, different to the hearing organ of vertebrate species, a nicotinic acetylcholine receptor composed only of α9 subunits operates at the LL efferent synapse. Activation of α9-containing (α9*) receptors leads to LL HC hyperpolarization due to the opening of Ca^{2+}-dependent potassium SK channels. These results will further aid in the interpretation of data obtained from LL HC as a model for cochlear HC.

Introduction

The processing of external stimuli is essential for all organisms to respond appropriately to environmental cues. Fishes and amphibians have a mechanosensory system, the lateral line (LL), which senses hydrodynamic information, crucial for behaviors such as obstacle and predator avoidance, schooling, prey capture and rheotaxis (Partridge and Pitcher, 1980; Bleckmann and Zelick, 2009; McHenry et al., 2009; Suli et al., 2012; Oteiza et al., 2017). The LL comprises cell clusters, called neuromasts, composed of mechanosensitive hair cells (HC) surrounded by non-sensory cells (Metcalfe et al., 1985). LL HC transmit sensory information to afferent neurons that project to the hindbrain (Metcalfe et al., 1985; Metcalfe, 1989; Liao, 2010). In addition, they are innervated by descending efferent fibers that modulate LL response to external stimuli (Metcalfe et al., 1985; Bricaud et al., 2001). Anatomical studies in fishes revealed two cholinergic efferent nuclei in the hindbrain, and a third dopaminergic nucleus in the forebrain (Hashimoto et al., 1970; Roberts and Russell, 1972; Zottoli and Van Horne, 1983; Tricas and Highstein, 1991; Bricaud et al., 2001). During movement, LL efferent cholinergic modulation aids the animal to distinguish between external and self-generated stimuli, maintaining sensitivity to relevant cues (Lunsford et al., 2019). Although it is known that D1b receptors mediate neurotransmission at the dopaminergic efferent synapse (Toro et al., 2015), this information is lacking for the cholinergic one.

In mammals, the best studied efferent-HC synapse, the cholinergic medial olivocochlear (MOC) efferent system makes direct synaptic contacts with HC. The net effect of MOC activity is to hyperpolarize HC (Guinan and Stankovic, 1996) through the activation of α9α10 nicotinic cholinergic receptors (nAChRs). Studies in heterologous systems and in HC of developing cochlear explants revealed the peculiar functional properties and high calcium (Ca^{2+}) permeability of α9α10 receptors (Elgoyhen et al., 2001; Gómez-Casati et al., 2005; Ballestero et al., 2011). Subsequent activation of Ca^{2+}-dependent SK2 potassium channels drives HC hyperpolarization (Dulon et al., 1998). Although similar molecules are probably expressed in all

vertebrate efferent synapses, this information is lacking for LL HC. However, what has been described for the receptors present at the mammalian efferent-HC synapses might not necessarily apply to other species, since the mammalian α9α10 nAChR has been under positive selection, rendering a receptor with unique functional properties (Franchini and Elgoyhen, 2006; Lipovsek et al., 2012; Marcovich et al., 2020).

LL HC share structural, functional and molecular similarities with those of the cochlea, making them a popular model for studying human hearing and balance disorders (Nicolson, 2005). In particular, efferent stimulation to the LL and the inner ear leads to inhibition of afferent transmission (Russell Ij, 1971; Roberts and Russell, 1972; Flock and Russell, 1976; Lunsford et al., 2019; Pichler and Lagnado, 2020), thus suggesting similar synaptic mechanisms. This is most likely brought about by cholinergic efferent fibers (Dawkins et al., 2005; Zhang et al., 2018) directly contacting the base of LL HC (Dow et al., 2018), similar to what has been described for MOC efferents. Moreover, similar to cochlear outer HC, LL HC have a postsynaptic cistern opposed to efferent terminals, proposed to participate in Ca^{2+} compartmentalization and/or Ca^{2+} induced Ca^{2+} release mechanisms (Lioudyno et al., 2004; Fuchs, 2014; Moglie et al., 2018; Zachary et al., 2018). These evidences suggest that the nAChR at the LL efferent synapse might be composed of α9 and α10 nAChR subunits.

To test this hypothesis we undertook a multi-pronged approach including the analysis of recent single-cell expression studies, cloning of zebrafish α9 and α10 nAChR subunits and profiling the biophysical and pharmacological properties of recombinant α9 and α9α10 receptors. In addition, we performed *in vivo* Ca^{2+} imaging of zebrafish LL HC to characterize the physiological signature of the native nAChR. We present strong evidence supporting the notion that the inhibitory signature of the LL efferent cholinergic synapse is most likely served by α9 homomeric receptors and the subsequent activation of Ca^{2+}-dependent SK potassium channels.

Materials and Methods

Cross-study evaluation of enriched gene expression in zebrafish hair cells

Pre-processed datasets from three single-cell RNAseq (Erickson and Nicolson, 2015; Matern et al., 2018, Lush et al., 2019) and one microarray (Steiner et al., 2014) studies that evaluated gene expression in HC from zebrafish were used (Table 1). Genes were searched by common name and accession number. For all data sets, we used the published normalized and batch-corrected gene relative expression quantification and calculated the differences between HC and control cells for each study as Log_2 Fold Change in order to perform comparisons among studies (Table 2). p-values adjusted for false discovery rate or q-values were obtained from each individual study. From the microarray dataset we used HC vs. mCh^+, GFP^+ mantle data. In the case of Lush et al. 2019, we used mature HC vs other neuromast cells (including immature HC).

Cloning of Zebrafish nAChR cDNAs

Zebrafish RNA was isolated from 7 days postfertilization (dpf) embryos using Trizol (Thermo Fisher scientific). mRNA was reverse transcribed using polyT primers with the SuperScript™ III First-Strand Synthesis System (Thermo Fisher scientific) to obtain whole embryo cDNA. Based on the sequences reported on the Genome Reference Consortium z11, specific primers were designed to amplify whole zebrafish α9 (ENSDARG00000054680) and α10 (ENSDARG00000011113) nAChR subunits cDNAs: α9 sense (5'- ATG AAG AGC AGT AGC AAA TAA TAA C -3'), α9 antisense (5'- AAT TGC AT AAG TTG TAA AC -3'); α10 sense (5'- ATG ATT TTA TAC TAT ATC C -3'), α10 antisense (5' TCA AAT GGC TTT CCC CAT TAT AAG -3'). 35 cycles were used in both cases with an annealing temperature of 45°C and 50°C for α9 and α10 subunits, respectively. PCR products were subcloned into pCR™2.1-TOPO® TA

160 vectors using TOPO TA Cloning Kit (Thermo Fisher scientific) and sequenced for verification of
161 correct amplification.
162
163 *Expression of Recombinant Receptors in Xenopus laevis Oocytes*
164 Zebrafish α9 and α10 cDNAs were sub-cloned into pSGEM vector, a modified pGEM-HE
165 vector suitable for *Xenopus laevis* oocyte expression studies (Liman et al., 1992). All expression
166 plasmids are readily available upon request. Capped cRNAs were transcribed *in vitro* using the
167 RiboMAX^{TM} Large Scale RNA Production System-T7 (Promega) from plasmid DNA templates
168 linearized with *NheI*. Both the maintenance of *X. laevis*, and the preparation and cRNA injection
169 of stage V and VI oocytes, has been described in detail elsewhere (Katz et al., 2000). Typically,
170 oocytes were injected with 50 nl of RNase-free water containing 0.01–1.0 ng of cRNAs and
171 maintained in Barth's solution at 18ºC. A 1 α9 : 2 α10 molar ratio was used to achieve
172 expression of the heteromeric receptor.
173 Electrophysiological recordings were performed 2– 6 days after cRNA injection under two-
174 electrode voltage clamp with a GeneClamp 500B Voltage and Patch Clamp amplifier (Molecular
175 Devices). Data acquisition was performed using a Digidata 1200 and pClamp 7.0 software
176 (Molecular Devices) at a rate of 10 points per second. Both voltage and current electrodes were
177 filled with 3M KCl and had resistances of ~0.5-2 MΩ. Data was analyzed using Clampfit from
178 the pClamp 7 software suite (Molecular Devices). During electrophysiological recordings,
179 oocytes were continuously superfused (10ml min^{-1}) with normal frog saline comprised of (mM):
180 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$ and 10 HEPES buffer, pH 7.2. Drugs were applied in the perfusion
181 solution of the oocyte chamber. V_{hold} was -70 mV except otherwise indicated.
182 In order to minimize the activation of the native oocyte's Ca^{2+}-sensitive chloride current
183 (ICl_{Ca}) by Ca^{2+} entering through nAChRs (Miledi and Parker, 1984; Boton et al., 1989), all
184 experiments were carried out in oocytes pre-incubated with the membrane permeant Ca^{2+}
185 chelator 1,2-bis (2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester

(BAPTA-AM; 100 μM) for 3 h prior to electrophysiological recordings, unless otherwise stated. This treatment was previously shown to effectively chelate intracellular Ca^{2+} ions and, therefore, to impair the activation of the ICl_{Ca} (Gerzanich et al., 1994). Concentration–response curves were obtained by measuring responses to increasing concentrations of ACh.

In order to assess whether Ca^{2+} ions are a major component of the inward current on nAChRs, we took advantage of the oocyte's endogenous ICl_{Ca} (Miledi and Parker, 1984; Boton et al., 1989) as an indirect reporter of Ca^{2+} entry through nAChRs. Current amplitudes were measured in normal frog saline, on the same oocyte before and after a 3-h incubation in BAPTA-AM. The percentage of the initial response remaining after BAPTA incubation was determined for each oocyte individually. Mean and S.E.M. of the percentage response after BAPTA was then determined for each receptor.

The effects of extracellular Ca^{2+} on the ionic currents through nAChRs were studied by measuring the amplitudes of the responses to a near-EC_{50} concentration of ACh (10 μM for α9 and 300 μM for α9α10) upon varying the concentration of this cation from nominally 0 to 3 mM at a holding potential of -90 mV (Weisstaub et al., 2002). Amplitude values obtained at each Ca^{2+} concentration were normalized to that obtained in the same oocyte at 1.8 mM. Values from different oocytes were averaged and expressed as the mean ± S.E.M. These experiments were carried out in oocytes injected with 7.5 ng of an oligonucleotide (5'-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3') antisense to connexinC38 mRNA (Arellano et al., 1995; Ebihara, 1996), in order to minimize the activation of the oocyte's nonselective inward current through a hemigap junction channel in response to the reduction of the external divalent cation concentration.

Desensitization of ACh-evoked currents was evaluated via a prolonged (1 min) agonist application, at a concentration one order of magnitude above the EC_{50} for each receptor. The percentage of current remaining 20s after the peak of the response was determined for each oocyte. Current-voltage (I-V) relationships were obtained by applying 2s voltage ramps from -

120 to +50mV from a holding potential of -70mV, at the plateau response to ACh (at a concentration one order of magnitude below the EC_{50} for each receptor). Leakage correction was performed by digital subtraction of the I–V curve obtained by the same voltage ramp protocol prior to the application of ACh.

Zebrafish husbandry and lines

Zebrafish (*Danio rerio*) were grown at 28.5°C on a light/dark cycle of 14:10 h, in E3 embryo medium (in mM: 130 NaCl, 0.5 KCl, 0.02 Na_2HPO_4, 0.04 KH_2PO_4, 1.3 $CaCl_2$, 1.0 $MgSO_4$, and 0.4 NaH_2CO_3). Embryos were obtained from natural spawning and bred according to guidelines outlined in The Zebrafish Book (Westerfield, 2000). For *in vivo* imaging and *in situ* hybridization experiments, 0.2 mM 1-phenyl2-thiourea (pTU) was added at 24 hours post fertilization (hpf) to prevent pigment formation. Animal experiments were done complying with the INGEBI institutional review board (Animal Care and Use Committee). Larvae were examined at 5 to 7 dpf unless otherwise stated. At these ages, sex cannot be predicted or determined, and therefore sex of the animal was not considered in our studies. For mRNA extraction and *in situ* hybridization studies wild-type fish of the AB strain were used. For *in vivo* Ca^{2+} imaging experiments, the double transgenic line Tg [Brn3c:Gal4] [UAS:GCaMP7a] (Xiao and Baier, 2007; Muto et al., 2013) was used.

Whole mount in situ hybridization

Embryos were fixed at 5 dpf in 4% paraformaldehyde overnight at 4°C, and stored at −20 °C in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). To avoid unwanted cross-reaction between nAChR genes, subunit specific probes were designed in non-conserved regions (intracellular loop of nAChR subunits) using the following primer sets: α9 sense 5'-TGAAAGTGATCGAGGCCCATT-3', α9 antisense 5'-TGTTTTCCACAGACACACCCTG-3', α10 sense 5'-GGACTGCAACTGCAACATGAA-3' and α10

antisense 5'-CACCCTTCCTGTCCTCTTCCT-3'. Partial sequences of genes of interest were PCR-cloned into pCR™2.1-TOPO® using Topo TA Cloning Kit (Thermo Fisher scientific) and used as templates to perform *in vitro* transcription to synthesize sense and antisense digoxigenin (DIG)- labeled probes. Sense probes were used as negative controls. Larvae were imaged on a Nikon Eclipse E200 microscope using a Nikon E Plan 10x/0.25 objective lens. Images were acquired via a Micrometrics® 891CU CCD 8.0 Megapixel camera using Micrometrics® SE Premium imaging software.

Sample preparation and stimulation for functional imaging

Individual Tg [Brn3c:Gal4;UAS:GcAMP7a] larvae at 5-7 dpf were first anesthetized with tricaine (0.03% ethyl 3-aminobenzoate methanesulfonate salt) and then pinned (through the head and tail) onto a Sylgard-filled recording chamber. To suppress movement, 125 μM α-Bungarotoxin (α-Btx) was injected directly into the heart. This technique is a suitable paralytic for LL recordings, since immunohistochemistry with antibodies against α-Btx confirmed labeling of muscle and did not label HC of the ear or neuromasts (Trapani and Nicolson, 2010). Larvae were then rinsed with extracellular imaging solution (in mM: 140 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH 7.3, OSM 310±10) without tricaine and allowed to recover. Viability was monitored by visually monitoring heart rate and blood flow.

Stimulation of neuromast HC was accomplished using a custom-made fluid jet. Pressure was applied using a 15 ml syringe and controlled through a TTL valve system (VC-6 valve controller, Warner instruments) triggered via the recording system. The output was attached to a glass pipette (inner tip diameter ~30–50 μm) filled with extracellular imaging solution and positioned parallel to the anterior–posterior axis of the fish in order to mechanically stimulate the apical bundles of HC along that axis (deflections were sustained for the duration of the stimulus, without flickering and kinocilial deflections were confirmed visually). We used the fluid jet to stimulate the HC of the two polarities by applying either negative or positive pressure. Air

volume injected through the syringe was constant (5 ml) and pressure was controlled with a manometer. Two second square stimuli were delivered in order to activate HC of all sensitivities (Zhang et al., 2018; Pichler and Lagnado, 2019).

Functional imaging

Fish were placed into a chamber on the stage of an upright microscope (Olympus BX51WI), illuminated with a blue (488 nm) LED system (Tolket) and images were acquired using an Andor iXon 885 camera controlled through a Till Photonics interface system. The focal plane was located close to the basal region of the neuromast in order to visualize the basal pole of HC. The signal-to-noise ratio was improved with a chip binning of 4x4, giving a resolution of 0.533 µm per pixel using a 60X water immersion objective. Acquisition rate was set to 6.6 frames/sec. Isradipine, α-Btx and apamin were applied in the bath and fish were pre-incubated prior to image acquisition (5 minutes in the case of Isradipine and 1 minute in the case of α-Btx and apamin). For ACh experiments, the drug was locally perfused throughout the whole image acquisition protocol (40 secs). In the case of co-application experiments, drugs were first pre-applied, as mentioned above, and then co-applied with ACh throughout the whole image acquisition protocol (40 secs). Fluorescence images were processed in FIJI (Schindelin et al., 2012; Rueden et al., 2017) and analyzed with custom-written routines in IgorPro 6.37 (Wavemetrics). Images were motion corrected using the StackReg plugin (Thévenaz et al., 1998). For all experiments, it was corroborated that the local perfusion did not elicit the activation of hair cells.

Regions of interest (ROIs) were hand-drawn for each visible HC in the neuromast. The mean ΔF/F 0 (%) was calculated in every ROI for each time frame and corrected for photobleaching by fitting a line between the pre-stimulus baseline and final fluorescence. Peak fluorescence signals were detected during the mechanical stimulation of the neuromast. Further analysis was performed if the peak signal was at least 2.5 standard deviations higher than the baseline. For

basal fluorescence intensity measurements, larvae were first imaged in extracellular imaging solution for 40 seconds, then exposed to ACh for 40 seconds and a second acquisition was taken. The mean basal fluorescence intensity was calculated and compared for every ROI before and during exposure to ACh.

Statistical analysis

For the biophysical characterization of recombinant receptors in *Xenopus laevis* oocytes all plotting and statistical tests were conducted using Prism 6 software (GraphPad Software Inc.). Concentration-response curves were normalized to the maximal agonist response in each oocyte. The mean and S.E.M. values of the responses are represented. Agonist concentration-response curves were iteratively fitted with the equation $I / Imax=[A]^n / ([A]^n+EC_{50}^n)$, where I is the peak inward current evoked by the agonist at concentration [A]; Imax is the current evoked by the concentration of agonist eliciting a maximal response; EC_{50} is the concentration of agonist inducing half-maximal current response and n is the Hill coefficient.

One-way repeated measures ANOVA, with a Geisser-Greenhouse correction to account for non-sphericity, was run to determine if there were statistical significant differences in responses to extracellular Ca^{2+}concentrations. A Bonferroni multiple comparison test was performed to evaluate differences between group means.

For *in vivo* Ca^{2+} imaging, all experiments were performed on a minimum of 8 animals (1 neuromast per animal) and on three independent days. Plotting and statistical analysis were performed using a custom written code in Python language (Python 3.7), using pandas, Scipy, numpy, IPython, matplotlib and seaborn packages (Hunter, 2007; Pérez and Granger, 2007; McKinney, 2010; Walt et al., 2011; Virtanen et al., 2020). Normality was tested using a Shapiro-Wilk normality test. As data was not normally distributed, statistical significance between two conditions was determined by Wilcoxon matched-pair ranks test. For this kind of analysis, effect sizes were calculated as Matched Pairs Rank Biserial Correlation (MPRBC), which equals the

316 simple difference between the proportion of favorable and unfavorable evidence (Kerby, 2014).
317 Statistical significance is reported at $\alpha = 0.05$
318
319 *Drugs*
320 All drugs were obtained from Sigma-Aldrich, except α-Btx and apamin that were purchased
321 from Alomone. For *in vivo* imaging experiments, drugs were brought to their final concentration
322 in normal extracellular imaging solution with 0.1% DMSO to improve basolateral drug
323 accessibility to LL HC in whole zebrafish larvae (Trapani and Nicolson, 2011; Sheets et al.,
324 2012, 2017; Toro et al., 2015; Zhang et al., 2018; Wong et al., 2019). Isradipine, apamin and α-
325 Btx were applied in the bath during pre-incubations and locally perfused. ACh was locally
326 perfused.

Results

Cross-study evaluation of enriched gene expression in zebrafish hair cells

In order to decipher the molecular players at the cholinergic efferent LL synapse, we first studied the expression of genes that encode key molecules of efferent synapses across vertebrates: chrna9 (gene encoding the α9 nAChR subunit), chrna10 (α10 subunit) and kcnn2 (small conductance Ca^{2+}-activated potassium channel 2, SK2), in LL HC. As there is evidence for the expression of other SK channels in zebrafish sensory organs (Cabo et al., 2013), we also evaluated the expression of kcnn1 and kcnn3 (genes encoding SK1 and SK3 channels, respectively). The Genome Reference Consortium Zebrafish Build 11 (GRCz11) indicates two ohnologues for kcnn1 (kcnn1a and kcnn1b) and only one copy for chrna9, chrna10, kcnn2 and kcnn3. A previous genome assembly version, GRCz10, had described two ohnologues for both chrna9 and chrna10 genes, but one of the copies of each gene (ENSDARG00000011029 and ENSDARG00000044353) have been deleted in GRCz11.

We collected data from recently published single-cell RNA-seq and microarray studies in zebrafish HC (Steiner et al., 2014; Erickson and Nicolson, 2015; Matern et al., 2018, Lush et al., 2019) (Table 1) and assessed the enrichment of chrna9, chrna10, kcnn1a, kcnn1b, kcnn2 and kcnn3 genes. Pre-processed data from each study (normalized, batch-corrected and with their adjusted p-values) was analyzed. The relative change in expression (Log_2 fold change) in HC was normalized to the control sample used in each study (Table 2).

Our analysis revealed that only chrna9, kcnn1a and kcnn2 transcripts are significantly enriched in HC (Table 2). These observations were true for all data sets analyzed in the case of chrna9 transcripts and in two of the four studies evaluated, in the case of kcnn1a and kcnn2. Surprisingly, chrna10 transcripts showed no enriched expression in HC (Table 2).

To further analyze the spatial expression pattern of α9 and α10 nAChRs subunits we performed whole mount *in situ* hybridization in 5 dpf larvae. To avoid unwanted cross-reaction

between nAChR genes, subunit specific probes were designed in the non-conserved intracellular loop of nAChR subunits. α9 subunit expression was localized to LL neuromasts and the posterior macula in the otic vesicle (Figure 1 A-E), confirming its expression in HC. However, due to spatial resolution, we cannot exclude the possibility that α9 is expressed in supporting cells too. No signal in neuromasts or the otic vesicle was detected for the α10 subunit mRNA (Figure 1 F), indicating that its expression level is null or under the detection limits of this technique. Moreover, we cannot rule out the possibility that the α10 subunit is expressed in LL HC at later developmental stages.

Biophysical and pharmacological characterization of zebrafish recombinant α9 and α9α10 nAChRs expressed in Xenopus laevis oocytes

To determine the possible combinatorial nAChR subunit assemblies leading to functional receptors and to analyze their pharmacological and biophysical properties, we performed RT-PCR with specific primers designed to isolate full-length zebrafish α9 and α10 nAChR subunit cDNAs, and subcloned them into pSGEM vector (a pGEM-HE vector optimized for *X. laevis* oocyte expression studies (Liman et al., 1992)). *In vitro* transcribed cRNAs were injected in *X. laevis* oocytes and responses to ACh were recorded under two-electrode voltage clamp.

Previous work reported that *Rattus norvegicus* (rat), *Xenopus tropicalis* (frog) and *Gallus gallus* (chicken) α9 subunits can form functional homomeric nAChRs. In contrast, only chicken and frog α10, but not rat subunits, assemble into functional homomeric receptors (Elgoyhen et al., 1994, 2001; Lipovsek et al., 2012, 2014; Marcovich et al., 2020). Whereas zebrafish α9 subunits assembled into functional homomeric receptors leading to robust ACh-evoked currents (Imax 425.52 ± 55.00 nA, n= 28), α10 subunits could not form functional receptors under our experimental conditions (Figure 2A). Oocytes injected with both α9 and α10 zebrafish cRNAs in an equimolar proportion responded to ACh in a concentration-dependent manner with a two component ACh dose-response curve that corresponds to both homomeric α9 and heteromeric

α9α10 nAChRs (Figure 2B). To favor the assembly of α9α10 heteromeric receptors and study its properties, we injected both cRNAs in a 1:2 α9:α10 ratio. ACh concentration-response curves for homomeric α9, α9α10 (1:1) and α9α10 (1:2) nAChRs are shown in Figure 2B. α9 nAChRs exhibited an EC_{50} of 11.71 μM (n=10; 95% CI 9.48 to 14.46), while the EC_{50} for α9α10 (1:2) receptors was 437 μM (n=7; 95% CI 357.2 to 534.6). It is interesting to note that, in contrast to that reported for rat receptors, α10 did not boost responses of the heteromeric α9α10 (Imax 292.05 ± 52.13 nA, n= 30), compared to the zebrafish homomeric α9 receptor.

Desensitization profile

A key feature of nAChRs is their desensitization after prolonged exposure to ACh (Quick and Lester, 2002). Zebrafish α9 and α9α10 receptors exhibited different desensitization profiles (Figure 2C). While in the case of the α9 nAChR a median of 44% (IQR: 40.36-50.75 %) of remaining current was observed 20 seconds after the peak response to 1 mM ACh, a median of 14.29% (IQR: 7.91-17.15 %) was observed for α9α10 receptors 20 seconds after the peak response to 3 mM ACh, indicating a faster desensitization (*p= 7.09e-06, U = 0.0, Mann-Whitney test).

Current-voltage relationship

Another distinctive feature that varies among α9α10 nAChRs is their current-voltage (I-V) relationship. Rat α9α10 receptors show a significant outward current at depolarized potentials and a greater inward current at hyperpolarized potentials (Elgoyhen et al., 2001). Chicken α9α10 nAChRs exhibit outward currents similar to their rat counterparts but smaller inward currents (Marcovich et al., 2020), and frog α9α10 receptors show an I-V profile with strong inward rectification and almost no outward current at depolarized potentials (Marcovich et al., 2020). Zebrafish α9α10 nAChRs also showed an unique I-V profile (Figure 2D), exhibiting considerable outward currents at depolarized potentials, similar to chicken α9α10 and rat α9

and α9α10 receptors (Elgoyhen et al., 2001). At hyperpolarized potentials, although with different amplitudes, both receptors exhibited inward rectification similar to their frog counterpart (Marcovich et al., 2020).

Ca^{2+} contribution to ACh-evoked responses

Ca^{2+} entry through α9α10 nAChRs is key for the function of the MOC-HC synapse, since the subsequent activation of Ca^{2+}-dependent potassium channels ultimately leads to the hyperpolarization of the HC. Ca^{2+} permeability of α9α10 nAChRs is not uniform across species (Lipovsek et al., 2012, 2014; Marcovich et al., 2020). In order to assess Ca^{2+} flux through zebrafish α9 and α9α10 nAChRs we analyzed the contribution of the *Xenopus* oocytes endogenous ICl_{Ca} (Miledi and Parker, 1984; Boton et al., 1989) to ACh-evoked responses. In oocytes expressing a recombinant receptor with high Ca^{2+} permeability, the ICl_{Ca} is strongly activated upon ACh application (Barish, 1983). Incubation of oocytes with the membrane-permeant fast Ca^{2+} chelator BAPTA-AM subsequently abolishes the Cl^- component of the total measured current (Gerzanich et al., 1994). Responses to ACh showed a strong reduction in peak amplitude after BAPTA incubation (Figure 3A, α9: 45.05 ± 5.87 % of peak current remaining, n = 15; α9α10: 38,97 ± 5.48 % of peak current remaining, n= 12), indicating a significant Ca^{2+} contribution to ACh-evoked responses for both receptors.

Modulation of ACh-evoked responses by extracellular Ca^{2+}

α9α10 nAChRs from rat, chicken, and frog exhibit differential modulation by extracellular Ca^{2+} (Weisstaub et al., 2002; Marcovich et al., 2020). Rat α9α10 receptors are both potentiated and blocked by extracellular Ca^{2+}, whereas in the case of frog and chicken α9α10 nAChRs ACh responses are only potentiated by this cation. Moreover, rat α9 receptors are only blocked by extracellular Ca^{2+}. To evaluate Ca^{2+} modulation of zebrafish α9 and α9α10 receptors, responses to near EC_{50} concentrations of ACh were recorded in normal Ringer's solution at different

431 extracellular Ca^{2+} concentrations and normalized to the response at 1.8 mM Ca^{2+}. Strikingly,
432 neither the homomeric α9 nor the heteromeric α9α10 nAChR responses to ACh were modulated
433 by extracellular Ca^{2+} (Figure 3B).
434
435 *Pharmacological characterization*
436 A hallmark of α9 and α9α10 nAChRs is their peculiar pharmacological profile, which includes
437 α-Btx and strychnine (Str) as potent inhibitors. We studied the effect of both drugs on α9 and
438 α9α10 nAChRs expressed in *Xenopus* oocytes. As shown in Figure 4A, pre-exposure of
439 oocytes for 1 min with 100 nM α-Btx before the co-application of 10 μM (α9) or 300 μM (α9α10)
440 ACh reduced agonist-evoked response by 94.59 ± 2.17% (n = 3) and 83.66 ± 5.50% (n= 3),
441 respectively. Similar block of ACh responses was obtained with strychnine (Figure 4B). Pre-
442 exposure of oocytes for 1 min with 1 μM Str before the co-application of 10 μM (α9) or 300 μM
443 (α9α10) ACh reduced agonist-evoked response by 95.44 ± 1.19% (n = 4) and 57.59 ± 5.1% (n=
444 3), respectively. In all cases the effect of α-Btx and Str were completely reversed by washing the
445 oocytes with frog saline solution for 5 min.
446
447 *In vivo functional Ca^{2+} imaging*
448 To characterize the physiological signature of the native nAChR present at the zebrafish LL
449 efferent synapse, we performed *in vivo* Ca^{2+} imaging in transgenic Tg
450 [Brn3c:Gal4;UAS:GcAMP7a] zebrafish larvae that specifically express the genetically encoded
451 Ca^{2+} sensor GcAMP7a in HC. We mechanically stimulated LL HC with saturating stimuli in both
452 rostral and caudal orientations, by applying positive and negative pressure through a pulled
453 glass pipette respectively, to elicit the activation of the mechanotransduction channel and thus
454 produce depolarization of HC and subsequent opening of voltage-gated Ca^{2+} channels (Cav
455 1.3a) (Zhang et al., 2018; Pichler and Lagnado, 2019). We measured peak fluorescence signals
456 derived from mechanically evoked HC depolarization and subsequent Ca^{2+} influx through

457 Cav1.3a channels as a proxy of the electrical state of HC (Figure 5A and B). As reported by
458 Wong et al., 2019, LL HC exhibited variable resting Ca^{2+} levels (655.9 ± 544.05 A.U., n=45).
459 Posterior LL HC are also variable in their function and signal transduction properties (Zhang et
460 al., 2018; Pichler and Lagnado, 2019). We therefore chose as our experimental unit single HC
461 that were selective for one polarity; that is HC that showed robust activation either by positive or
462 negative deflections (e.g cells 1,2,3,4,5,8 and 9 in Figure 5B). HC exhibited robust and stable
463 Ca^{2+} signals over two trials with the same stimulation after 1 minute (Figure 5C, 1° stim: Mdn
464 ΔF/F0= 0.858 IQR: 0.472-1.504 Vs. 2° stim: Mdn ΔF/F0= 0.876 IQR: 0.475-1.503, n= 113, W= -
465 835, p= 0.2317, matched pairs rank biserial correlation (MPRBC)= 0.129 Wilcoxon matched-
466 pairs signed rank test). Consistent with previous findings (Sheets et al., 2012; Zhang et al.,
467 2018), HC pretreated with the Cav1.3 antagonist isradipine (10 μM) showed a significant
468 decrease in Ca^{2+} entry levels with respect to control conditions (Figure 5D, Ctrl: Mdn ΔF/F0=
469 0.743 IQR: 0.249-1.086 Vs. Isr: Mdn ΔF/F0= 0.209 IQR: 0.079-0.433, n = 23, W= -258, p =
470 8.726e-05, MPRBC= 0.935; Wilcoxon matched-pairs signed rank test). This finding reveals that
471 under our experimental conditions a large proportion of the total change in fluorescence
472 intensity after mechanical stimulation can be attributed to Ca^{2+} influx through Cav1.3a channels.
473 The remaining change in fluorescence intensity is most likely due to Ca^{2+} entering through the
474 mechanotransduction channel (Zhang et al., 2018).

475 Stimulation of cholinergic efferents in the LL of *Xenopus*, burbot *Lota lota*, and dogfish
476 *Scyliorhynus*, inhibits spontaneous and evoked activity of afferents by generating inhibitory
477 postsynaptic potentials in HC (Russell Ij, 1971; Roberts and Russell, 1972; Flock and Russell,
478 1976). In zebrafish, activation of cholinergic efferents suppresses glutamate release from HC
479 (Pichler and Lagnado, 2020) and inhibits afferent activity (Lunsford et al., 2019). This most likely
480 results from ACh-evoked hyperpolarization of HC and reduced Ca^{2+} influx through voltage-
481 activated Ca^{2+} channels.

In order to analyze the effect of nAChR activation on HC, we evaluated the change in fluorescence intensity on mechanically-stimulated HC pretreated with ACh. If the activation of nAChR leads to HC hyperpolarization, then exogenous application of ACh should result in a decreased change in fluorescence intensity due to a reduced activation of Cav1.3 channels and thus lower Ca^{2+} influx. As expected, exogenous application of 1 mM ACh on mechanically-stimulated HC elicited a significant decrease in evoked Ca^{2+} influx with respect to control (Figure 6A and B, Ctrl: Mdn ΔF/F0= 0.818 IQR: 0.341-1.479 Vs. ACh: Mdn ΔF/F0= 0.573 IQR: 0.315-1.101, n = 114, W= -3493, p = 7.89e-07, MPRBC = 0.532, Wilcoxon matched-pairs signed rank test). This effect was reversed by superfusing the preparation with extracellular imaging solution (Figure 6C, n= 37, Friedman test, Q= 18.54, p=9.418e-05. Dunn's multiple comparisons test, Extra Vs. ACh: p=0.000705, Extra Vs. Wash: p=0.608054). ACh application *per se* did not evoke changes in basal fluorescence intensity (Figure 6D, naïve: 655.9 ± 81.1 Arbitrary Units (A.U.) vs. ACh: 652.2 ± 78.55 A.U, n = 45 cells, t= 0.7816, df= 44, p = 0.4386, two-tailed paired t-test). Furthermore, ACh mediated effect was observed in HC irrespective of polarity. Figure 6E shows that exogenous application of ACh elicited a significant decrease in mechanically-evoked Ca^{2+} influx, both in HC selectively activated by an anterior to posterior (Ant-Post) or by a posterior to anterior (Post-Ant) stimulus (Ant-Post, Extra Mdn ΔF/F0= 0.783 - ACh Mdn ΔF/F0= 0.559, n= 62, W= -1227, p= 1.698e-05, MPRBC= 0.628; Post-Ant, Extra Mdn ΔF/F0= 0.831 - ACh Mdn ΔF/F0= 0.653, n= 52, W= -582, p=0.008, MPRBC= 0.422; Wilcoxon matched-pairs signed rank test). In addition, there was no significant difference in the magnitude of ACh mediated-effect between HC with different polarity selectivity (Figure 6F, (Ant-Post Mdn rel. ΔF/F0 diff = -0.2003 IQR: -0.424-0.051 Vs. Post-Ant Mdn rel. ΔF/F0 diff = -0.241 IQR: -0.497-0.118, U= 1560, p = 0.7687; Mann-Whitney test). Noteworthy, ACh-mediated effect on evoked Ca^{2+} signals was heterogeneous. To quantify the degree of inhibition elicited by ACh we used an *ad-hoc* metric, the Inhibition Index (*II*) that was calculated for each HC in Figure 6A. If

507 $\Delta F/F0_{extra}$ is the change in fluorescence intensity on mechanically-stimulated HC under control
508 conditions and $\Delta F/F0_{ACh}$ is the change in fluorescence intensity on mechanically-stimulated HC
509 pretreated with ACh, then *II* was calculated as:

$$II = \frac{\frac{\Delta F}{F0_{Extra}} - \frac{\Delta F}{F0_{Ach}}}{\frac{\Delta F}{F0_{Extra}}}$$

510 Thus, *II*=0 indicates no inhibition, *II*=1 indicates full inhibition and 0 <*II*<1 indicates partial
511 inhibition. Figure 6G shows the distribution of *II* for ACh-treated HC. As expected, in the majority
512 of cases *II* values were > 0. However, a subpopulation of cells exhibited *II* values close to 0,
513 denoting no ACh-mediated inhibition. The absence of ACh-treated HC with *II*=1 is a
514 consequence of our selection criteria, that is selecting cells with measurable mechanically-
515 evoked Ca^{2+} signals in both control conditions and during ACh treatment. Consequently, the
516 inhibitory effect of ACh might be underestimated.

517 To assess the identity of the nAChR mediating synaptic transmission at the LL efferent
518 synapse, we tested the effect of α-Btx, a potent inhibitor of recombinant zebrafish α9 and α9α10
519 nAChRs, on ACh-mediated inhibition of evoked Ca^{2+} signals. Figure 7B shows that ACh
520 modulation was blocked when this agonist was co-applied with 10 μM α-Btx (α-Btx: Mdn ΔF/F0=
521 0.534 IQR: 0.3308-1.325 Vs. ACh-α-Btx: Mdn ΔF/F0= 0.4015 IQR: 0.203-0.816, n = 25, W= -87,
522 p= 0.2541, MPRBC = 0.268, Wilcoxon matched-pairs signed rank test), supporting the
523 hypothesis that a functional α9* nAChR is present at the zebrafish LL efferent synapse.

524 In mammals and birds, the inhibitory sign of the efferent synapse is due to the entry of Ca^{2+}
525 through α9α10 receptors and the subsequent activation of a small-conductance SK2 Ca^{2+}-
526 dependent potassium channel (Hiel et al., 2000; Oliver et al., 2000; Gómez-Casati et al., 2005;
527 Matthews et al., 2005; Elgoyhen and Katz, 2012). To evaluate the coupling of ACh responses to
528 SK activation in zebrafish LL efferent synapse, we analyzed the effect of apamin, a known SK
529 channel blocker, on ACh-mediated inhibition of evoked Ca^{2+} signals. Co-application of 1 mM

ACh and 10 μM apamin abolished the inhibitory effect of ACh (Figure 7D, Apa: Mdn ΔF/F0= 0.470 IQR: 0.176-0.818 Vs. Apa-ACh : Mdn ΔF/F0= 0.508 IQR: 0.192-0.718, n = 60, W= -322, p= 0.2359, MPRBC = 0.1759, Wilcoxon matched-pairs signed rank test), suggesting that the nAChR that serves the LL efferent synapse is functionally coupled to an SK channel.

Discussion

Vertebrate HC systems are innervated by efferent fibers that modulate their response to external stimuli (Russell, 1971; Metcalfe et al., 1985; Guinan and Stankovic, 1996; Bricaud et al., 2001). In the LL, the excitation of efferent fibers inhibits afferent activity by generating inhibitory postsynaptic potentials in HC (Russell, 1971; Flock and Russell, 1973, 1976). In addition, excitatory efferent effects, mediated by dopamine acting through D1b receptors (Toro et al., 2015), can be observed when cholinergic transmission is blocked (Flock and Russell, 1973). To date, the molecular players serving ACh-mediated inhibitory effects remain unknown. Here we provide evidence for a mechanism in which an α9* nAChR operates at the zebrafish LL efferent synapse. Our study suggests that Ca^{2+} influx through these receptors activates nearby SK channels, leading to LL HC hyperpolarization (Figure 8).

Efferent innervation mediated by α9* nAChRs is a common feature to all known vertebrate HC (Elgoyhen et al., 1994; Glowatzki and Fuchs, 2000; Hiel et al., 2000; Holt et al., 2003; Parks et al., 2017). In mammals, MOC efferent activity is mediated by α9α10 nAChRs (Elgoyhen et al., 2001; Lustig et al., 2001; Sgard et al., 2002; Gómez-Casati et al., 2005). Moreover, α10 subunits are strictly required for efferent function, since α9 nAChRs expressed in α10−/− mice are unable to drive efferent signals (Vetter et al., 2007). Surprisingly, our analysis of previous single-cell studies (Table 2) and *in situ* hybridization data showed enriched expression of α9 but not α10 subunits in zebrafish HC. Furthermore, our functional data proved that zebrafish α9 nAChRs expressed in *Xenopus* oocytes exhibit robust ACh-evoked currents which are not boosted in magnitude when co-expressed with α10. This is in stark contrast to that observed for heterologously expressed rat α9 receptors which exhibit very small ACh-evoked responses, that are non-reliable and significantly boosted when co-expressed with α10 (Elgoyhen et al., 1994, 2001; Sgard et al., 2002). Therefore, our expression and functional data strongly suggest that an α9 homomeric nAChR operates at the LL efferent synapse.

559 Striking features of the zebrafish α9 nAChR are its high desensitization rate and lack of
560 modulation by external Ca^{2+}. This differs from that reported for rat (Elgoyhen et al., 1994; Katz
561 et al., 2000) and chicken (Lipovsek et al., 2012) α9 receptors, since both exhibit low
562 desensitization kinetics, and rat receptors (not reported for chicken) are blocked by extracellular
563 Ca^{2+}. These results support the observation that within the nAChR family, α9 and α10 subunits
564 exhibit the highest degree of coding sequence divergence, mirrored by a great variability of
565 functional properties across species (Franchini and Elgoyhen, 2006; Lipovsek et al., 2012;
566 Marcovich et al., 2020).

567 Differences in nAChR abundance and/or localization and anatomical arrangement of efferent
568 terminals contacting HC might exist between fish and mammals. However, as reported for
569 cochlear HC (Moglie et al., 2018), Ca^{2+} homeostasis is probably a key feature of LL efferent-HC
570 synapses. The high desensitization kinetics of zebrafish α9* receptors probably leads to a self-
571 limiting Ca^{2+} entry through this highly Ca^{2+} permeable nAChR. As reported for developing inner
572 HC (Moglie et al., 2018), this is key to prevent crosstalk between efferent and afferent systems,
573 which co-exist in LL HC, and could lead to Ca^{2+} spillover from efferent-mediated Ca^{2+} entry to
574 Ca^{2+}-triggered glutamate release and activation of afferent fibers. Furthermore, as for cochlear
575 HC (Moglie et al., 2018), postsynaptic cisterns opposed to LL efferent terminals (Dow et al.,
576 2018) could also aid to prevent efferent-to-afferent cross-talk. In rat and chicken, the
577 desensitization capability of α9α10 receptors is provided by the α10 subunit (Elgoyhen et al.,
578 2001; Lipovsek et al., 2012). Since in zebrafish LL the efferent response most likely relies on α9
579 nAChRs, one could propose that substitutions in the coding sequence of this subunit might have
580 led to a receptor highly fitted to convey self-limiting Ca^{2+} influx into HC, and this should be
581 further tested.

582 The increase in intracellular Ca^{2+} upon deflection of the cilia, results from Ca^{2+} influx through
583 mechanosensitive ion channels (Corey and Hudspeth, 1979; Fettiplace, 2009; Zhang et al.,
584 2018) and the subsequent activation of voltage-gated Ca^{2+} channels due to HC depolarization

(Moser and Beutner, 2000; Sheets et al., 2017; Zhang et al., 2018). The fact that the application of ACh resulted in a reduction of Ca^{2+} signals, most likely indicates a reduced depolarization. Thus one could propose that ACh inhibits Ca^{2+} influx due to a net hyperpolarization of LL HC. The finding that ACh-mediated effect can be blocked by apamin, supports the generally held hypothesis that Ca^{2+} entering through the efferent nAChR activates nearby SK channels leading to HC hyperpolarization (Doi and Ohmori, 1993; Blanchet et al., 1996; Nenov et al., 1996; Yuhas and Fuchs, 1999; Glowatzki and Fuchs, 2000; Oliver et al., 2000; Holt et al., 2003, 2003; Katz et al., 2004; Dawkins et al., 2005; Gómez-Casati et al., 2005; Parks et al., 2017). In birds (Matthews et al., 2005) and mammals (Dulon et al., 1998; Oliver et al., 2000), α9* nAChRs are functionally coupled to SK2 K^+ channels. However our cross-study analysis revealed that both kcnn2 (SK2) and kcnn1b (SK1b) transcripts are enriched in zebrafish HC. This is consistent with Cabo et al. (2013) which showed SK1 expression in zebrafish LL HC. Interestingly, SK1 and SK2 are generally co-expressed in the brain of fish (Ellis et al., 2008) and mammals (Stocker and Pedarzani, 2000). Moreover, rat SK1 forms functional heteromeric channels with SK2 (Benton et al., 2003; Autuori et al., 2019). The fact that apamin blocked ACh-mediated effects suggests that SK2 channels play a key role in zebrafish LL HC hyperpolarization, since these channels are the most apamin-sensitive (Köhler et al., 1996; Shah and Haylett, 2000; Strøbaek et al., 2000; Stocker, 2004).

Zebrafish neuromasts contain two populations of HC which are activated by deflections in either the anterior or posterior direction (Flock and Wersall, 1962; Ghysen and Dambly-Chaudiere, 2007). However, only one efferent fiber contacts all HC of a single neuromast (Faucherre et al., 2009; Dow et al., 2018). Moreover, during fictive locomotion presynaptic activity across all efferent synapses within a neuromast are synchronously activated (Pichler and Lagnado, 2020). Similarly, in our experiments ACh-mediated effects were observed in HC irrespective of their polarity and the median magnitude of inhibition in both cases showed no significant difference. However, Pichler and Lagnado (2020) reported that efferent modulation is

biased toward HC activated during forward motion. This discrepancy might rely on the fact that our experiments were performed perfusing ACh and not by stimulation of efferent terminals (Pichler and Lagnado, 2020). Differences in the efficiency of presynaptic ACh release at efferent terminals and/or in the number of efferent terminals per HC of different polarities might account for this biased efferent modulation. Alternatively, physiological heterogeneity of LL HC could contribute to differences in the efficiency with which depolarization triggers glutamate release.

HC within the same neuromast exhibit functional heterogeneity. Stimuli able to open mechanosensitive channels are insufficient to evoke vesicle fusion in the majority of HC (Zhang et al., 2018). Moreover, synaptically active HC exhibit lower intracellular K^+ (Kin) levels than silent HC. We show that resting Ca^{2+} levels are heterogenous too, as reported by Wong et al. (2019). This variability could arise from differences in the expression level of GCaMP7 or from heterogeneity among HC. Hair cells with lower Kin might be at sufficiently depolarized resting membrane potentials to activate CaV1.3 channels following spontaneous opening of mechanotransduction channels, resulting in higher resting Ca^{2+} levels. In contrast, HC with higher Kin levels would exhibit lower resting Ca^{2+} levels. Furthermore, heterogeneity has also been shown for LL afferent response to efferent activity (Lunsford et al., 2019). In tune with these findings, we show that the ACh-mediated effect on evoked Ca^{2+} signals is heterogeneous, adding a new level of complexity underlying LL HC function *in vivo*. Differences in the density of α9* nAChRs and/or SK channels could explain this phenomenon. Alternatively, ACh accessibility to HC depending on their location within the neuromast, may also contribute to this variability.

The LL system controls many behaviors such as schooling (Partridge and Pitcher, 1980; Mekdara et al., 2018), prey capture (McHenry et al., 2009; Stewart et al., 2013) and rheotaxis (Bleckmann and Zelick, 2009; Olszewski et al., 2012; Suli et al., 2012; Oteiza et al., 2017). However, the role of the efferent system on the performance of these behaviors remains unknown. Deciphering the molecular players at the zebrafish cholinergic LL efferent synapse

637 will enable the generation of molecular tools to selectively manipulate its activity and evaluate its
638 role on sensory processing and associated behaviors in their native context. In addition, due to
639 the overall similarity between mammalian and piscine efferent synapses, zebrafish emerges as
640 an excellent platform to evaluate compounds that target α9* nAChRs to treat pathologies such
641 as noise-induced hearing loss and tinnitus.

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936

Figure 1 α9 (but not α10) is expressed in zebrafish LL neuromasts and the posterior macula in the otic vesicle

Whole-mount *in situ* hybridization with antisense (A, B, C and D) and sense (E) α9, and antisense α10 (F) riboprobes. Representative lateral views with anterior to the left and dorsal to the top, are shown. Arrow indicates the otic vesicle and arrowheads point to selected neuromasts. Large scale view of the otic vesicle (**C**) and neuromasts (**B** and **D**). (C) Dotted line delimits the otic vesicle; dotted-dashed line outlines the posterior macula (pm). dpf, days post-fertilization. Scale bars: 100 μm in **A, E** and F, 40 μm in **B**, 25 μm in **C**, 10 μm in **D**.

Figure 2 Zebrafish recombinant α9 forms homomeric and heteromeric receptors with α10 with distinct biophysical properties

A, Representative responses evoked by ACh in oocytes expressing either zebrafish α9, α10 or α9α10 (1:2) nAChRs. **B**, Concentration-response curves for zebrafish α9, α9α10 (1:1) and α9α10 (1:2) nAChRs. Values are the mean ± S.E.M. Lines are best fit to the Hill equation. **C**, *Top*, Representative responses of zebrafish α9 and α9α10 (1:2) nAChRs to a 60 s application of ACh (1 order of magnitude higher than their corresponding EC_{50}); *Bottom*, desensitization rate shown as percentage of current remaining 20s after the peak response relative to the maximum current amplitude elicited by ACh. Lines indicate the median and IQR. Symbols represent individual oocytes (n= 13 and 9, respectively). **D**, Representative I-V curves obtained by the application of voltage ramps (-120 to +50 mV, 2s) at the plateau response to 10 μM ACh for both zebrafish α9 and α9α10 (1:2) nAChRs. Values were normalized to the agonist response at +50 mV for each receptor.

Figure 3. Zebrafish α9 and α9α10 nAChRs have a high Ca^{2+} contribution to the total inward current and are not modulated by extracellular Ca^{2+}

962 **A**, *Top*, Representative responses to a near-EC_{50} concentration of ACh (10 μM for α9 and 300
963 μM for α9α10) in oocytes expressing zebrafish α9 and α9α10 nAChRs before (light colors) and
964 after (solid colors) a 3-h incubation with BAPTA-AM. *Bottom*, Percentage of the initial peak
965 response remaining after BAPTA-AM incubation. Lines indicate the median and IQR. Symbols
966 represent individual oocytes (n= 14 and 8, respectively), **B**, ACh response amplitude as a
967 function of extracellular Ca^{2+} concentration (*Top*, α9; *Bottom*, α9α10). ACh was applied at a
968 near EC_{50} concentration (10 μM for α9 and 300 μM for α9α10). Current amplitudes recorded at
969 different Ca^{2+} concentrations in each oocyte were normalized to the response obtained at 1.8
970 mM Ca^{2+} in the same oocyte (α9, gray circles; α9α10, pink circles). Vhold: -90 mV. Bars
971 represent mean ± S.E.M (α9, black bars, n= 8; α9α10, red bars, n=5).

972

973 **Figure 4**. Zebrafish α9 and α9α10 nAChRs are reversibly blocked by α-Btx and Strychnine
974 Responses to 10 μM (α9) or 300 μM (α9α10) ACh either alone, in the presence of α-Btx (A) or
975 Str (B), or after washing with control bath solution for 5 min, in oocytes expressing zebrafish α9
976 or α9α10 nAChRs are shown. (A) Oocytes were pre-incubated with 100 nM α-Btx for 1 min prior
977 to the addition of the agonist. α-Btx inhibited ACh-elicited responses through α9 nAChRs by
978 94.59 ± 2.17% (n = 3) and through α9α10 nAChRs by 83.66 ± 5.50% (n= 3). (B) Oocytes were
979 pre-incubated with 1 μM Str for 1 min prior to the addition of ACh. Str inhibited ACh-evoked
980 currents through α9 nAChRs by 95.44 ± 1.19% (n = 4) and through α9α10 nAChRs by 57.59 ±
981 5.1% (n = 3).

982

983 **Figure 5.** Mechanical stimulation elicits a robust Ca^{2+} signal that is inhibited by isradipine
984 **A**, Representative functional Ca^{2+} images of a double transgenic neuromast expressing
985 GcAMP7a in HC. **i** Pre-stimulus baseline grayscale image (ROIs are drawn around each visible
986 hair cell), **ii** and **iii** Spatial patterns of GCaMP7a Ca^{2+} signals, during a 2 sec mechanical

stimulus in either the anterior-posterior (→) or in the posterior-anterior direction (←), are color coded according to the ΔF/F0 heat map. **B**, Representative temporal curves of mechanosensitive Ca^{2+} responses (ΔF/F0) of HC numbered in **A**, normalized to the peak intensity for each cell. Shaded areas indicate the time when the neuromast was mechanically stimulated. **C**, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, over two trials with same stimulation after 1 minute (1° stimulus light red, 2° stimulus dark red). Curves are aligned to the onset of the mechanical stimulus. *Bottom,* Peak ΔF/F0 for single HC (n = 113, each in its preferred orientation) over two trials with the same stimulation 1 minute apart. **D**, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, before (red) and after (purple) pre-incubation with 10 μM isradipine. Curves are aligned to the onset of the mechanical stimulus. *Bottom,* Pre-incubation with 10 μM isradipine drastically reduced peak ΔF/F0 (n = 23, W= -258, *p = 8.726e-05, MPRBC= 0.935; Wilcoxon matched-pairs signed rank test). Extra: Extracellular imaging solution, Isr: Isradipine. Scale bar in **A**: 5 μm. Horizontal scale bar in **C** and **D:** 1.5 sec, Vertical scale bar in **C** and **D**: 25% ΔF/F0. Duration of the stimulus in **C** and **D** *Top* is indicated by gray lines below each trace.

Figure 6. ACh inhibits mechanically-evoked Ca^{2+} signals and this inhibition is heterogeneous and independent of HC polarity

A, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, over two trials with the same stimulation 1 minute apart (1° stimulus (light red), 2° stimulus (dark red)). Curves are aligned to the onset of the mechanical stimulus. *Bottom,* Peak ΔF/F0 for single HC (n = 113) over two trials with the same stimulation after 1 minute. **B**, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, before (red) and after (blue) the application of 1 mM ACh. Curves are aligned to the onset of the mechanical stimulus. *Bottom*, ACh application reduces mechanosensitive Ca^{2+} responses (n = 114, W= -3493, *p =

7.89e-07, MPRBC = 0.532, Wilcoxon matched-pairs signed rank test). **C**, ACh-mediated reduction in mechanically-evoked Ca^{2+} signals is reversed after 1 minute wash with extracellular imaging solution. (n= 37, Friedman test, F= 18.54, p=9.418e-05, Dunn's multiple comparisons test, Extra Vs. ACh: p=0.000705, Extra Vs. Wash: p=0.608054). **D**, Basal Ca^{2+} levels show no significant differences before and during the application of 1 mM ACh (n = 45 cells, t= 0.7816, df= 44, p = 0.4386, two-tailed paired t-test). **E**, ACh reduces mechanosensitive Ca^{2+} responses in HC of opposing polarity (Ant-Post, n= 62, W= -1227, *p= 1.698e-05, MPRBC= 0.628; Post-Ant, n= 52, W= -582, *p=0.008, MPRBC= 0.422; Wilcoxon matched-pairs signed rank test). **F**, HC of opposing polarity exhibit no significant differences between their ACh-mediated relative change in peak ΔF/F0. (U= 1560, p = 0.7687; Mann-Whitney test). **G**, Distribution of Inhibition Index (*II*, calculated as (ΔF/F0extra - ΔF/F0ACh) / ΔF/F0extra) for ACh-treated HC. *Inset*, Distribution of Change Index (*CI*, calculated as (ΔF/F0stim1 - ΔF/F0stim2) / ΔF/F0stim1) for two successive mechanical stimuli under control conditions. The distribution of *CI* is centered around 0. A reduced number of cells (<10%) exhibit large negative *CI* values that occur when fluorescence signal is greater during the 2° stimulus, suggesting these might be outliers. Lines indicate the median and IQR. Extra: Extracellular imaging solution, ACh: Acetylcholine, Ant - Post: Anterior - Posterior deflection preference. Post - Ant: Posterior - anterior deflection preference. Horizontal scale bar in **A** and **B**: 1.5 sec, Vertical scale bar in **A** and **B**: 25% ΔF/F0. Duration of the stimulus in **A** and **B** *Top* is indicated by gray lines below each trace.

Figure 7 ACh-mediated inhibition of evoked Ca^{2+} signals is blocked by α-Btx and apamin.

A, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, before (red) and after the application of 10 μM α-Btx (green). *Bottom*, Mechanosensitive Ca^{2+} signals show no significant difference before and after 10 μM α-Btx treatment (Extra: Mdn ΔF/F0= 0.509 IQR: 0.252-1.134 Vs. α-Btx: Mdn ΔF/F0= 0.534 IQR: 0.331-1.325, n = 25, W= -45, p= 0.5449, MPRBC = 0.138). **B**, *Top*, Representative temporal ΔF/F0 curves of

mechanosensitive Ca^{2+} responses of 4 HC, after the application of 10 μM α-Btx (green) and after the co-application of 1 mM ACh and 10 μM α-Btx (blue). *Bottom*, When co-applied with 10 μM α-Btx, ACh-mediated inhibition is blocked (n = 25, W= -87, p= 0.2521, MPRBC = 0.268). **C**, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 hair cells, before (red) and after the application of 10 μM apamin (orange). *Bottom*, Mechanosensitive Ca^{2+} signals show no significant difference before and after 10 μM apamin treatment (Extra: Mdn ΔF/F0= 0.599 IQR: 0.243-1.216 Vs. Apa: Mdn ΔF/F0= 0.567 IQR: 0.191-1.040, n = 41, W= -91, p= 0.5554, MPRBC = 0.106). **D**, *Top*, Representative temporal ΔF/F0 curves of mechanosensitive Ca^{2+} responses of 4 HC, after the application of 10 μM apamin (orange) and after the co-application of 1 mM ACh and 10 μM apamin (blue). *Bottom*, ACh-mediated inhibition is blocked by 10 μM apamin (n = 60, W= -322, p= 0.2359, MPRBC = 0.1759). A Wilcoxon matched-pairs signed rank test was used in all cases. Extra: Extracellular imaging solution, ACh: Acetylcholine, α-Btx: α-bungarotoxin, Apa: Apamin. Horizontal scale bar in **A**, **B**, **C** and **D**: 1.5 sec, vertical scale bar in **A**, **B**, **C** and **D**: 25% ΔF/F0. Curves in A, B, C and D are aligned to the onset of the mechanical stimulus. Duration of the stimulus is indicated by gray lines below each trace.

Figure 8. Schematics of the cholinergic LL efferent synapse.

LL HC are innervated by afferent (red) and cholinergic efferent (green) fibers. Evidence for efferent cholinergic fibers contacting afferent neurons (dashed light green) is still missing. The net effect of LL efferent cholinergic activity is to hyperpolarize HC. This is mediated by the activation of an α9* nAChR with high Ca^{2+} permeability. Subsequent activation of Ca^{2+}-dependent potassium SK channels drives HC hyperpolarization. Postsynaptic cisterns (PC) opposed to efferent terminals (Dow et al., 2018) have been proposed to participate in Ca^{2+} compartmentalization and/or Ca^{2+} induced Ca^{2+} release mechanisms.

Table 1. Meta-data of the studies used to analyze gene enrichment in zebrafish HC

Table 2. α9 (but not α10), SK1a and SK2 are enriched in zebrafish HC.

Log_2 Fold Change and adjusted p-values for the genes of interest across the different studies analyzed. (+) indicates a gene that shows enrichment of its expression in HC in at least one of the studies. (*) indicates a significant adjusted p-value.

1070 **Table 1**

Publication	DOI	Age (dpf)	HC promoter	Sorting method	Control	Expression level by
Steiner, et al. 2014	10.1073/pnas.1318692111	4	pou4f3	FACS	Skin	Microarray
Erickson, et al. 2015	10.1186/s12864-015-2072-5	3.5	myo6b	TU-tagging	Non-TU cells	RNA-Seq
Matern, et al. 2018	10.3389/fcell.2018.00047	5	myo6b	RiboTag	Whole larva	RNA-Seq
Lush, et al. 2019	10.7554/eLife.44431	5	pou4f3	FACS	Neuromast non-HC	RNA-Seq

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1072 **Table 2**

		Matern et al. (2018)		Erickson et al. (2015)		Steiner et al. (2014)		Lush et al. (2019)	
Gene	Ensembl ID	Log_2 FC	adj p-val.	Log_2 FC	adj p-val.	Log_2 FC	adj p-val.	Log_2 FC	adj p-val.
Chrna9(+)	ENSDARG00000054680	3.655976	8.05513E-13(*)	2.08078	0.040471217(*)	4.239971	4.78929E-11(*)	0.892044	7.827853E-116(*)
chrna10	ENSDARG00000011113	0.162382	1	-0.15769	1	0.03275	0.849872	-	-
Kcnn1a(+)	ENSDARG00000091306	1.34144	0.001182439(*)	-0.46363	1	0.789479	0.0218807(*)	-	-
Kcnn1b	ENSDARG00000023546	0.120795	1	0.2286	1	0.11828	0.565259	-	-
Kcnn2(+)	ENSDARG00000014939	1.484557	0.000490792(*)	0.151657	1	3.958601	5.91E-06(*)	-	-
kcnn3	ENSDARG00000019753	-0.96006	0.413984684	-0.44232	1	0.096667	0.60571	-	-

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