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Cholinergic modulation of time-coding neurons

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Abstract

Sensory processing in the lower auditory pathway is generally considered to be rigid and thus less subject to modulation than central processing. However, in addition to the powerful bottom-up excitation by auditory nerve fibers, the ventral cochlear nucleus also receives efferent cholinergic innervation from both auditory and non-auditory top-down sources. We thus tested the influence of cholinergic modulation on highly-precise time-coding neurons in the cochlear nucleus of the Mongolian gerbil. By combining electrophysiological recordings with pharmacological application in vitro and in vivo, we found 55-72% of spherical bushy cells (SBC) to be depolarized by carbachol on two time scales, ranging from hundreds of milliseconds to minutes. These effects were mediated by nicotinic and muscarinic acetylcholine receptors, respectively. Pharmacological block of muscarinic receptors hyperpolarized the resting membrane potential, suggesting a novel mechanism of setting the resting membrane potential for SBC. The cholinergic depolarization led to an increase of spike probability in SBC without compromising the temporal precision of the SBC output in vitro. In vivo, iontophoretic application of carbachol resulted in an increase in spontaneous SBC activity. The inclusion of cholinergic modulation in an SBC model predicted an expansion of the dynamic range of sound responses and increased temporal acuity. Our results thus suggest of a top-down modulatory system mediated by acetylcholine which influences temporally precise information processing in the lower auditory pathway.

Significance statement

Information processing in sensory neural pathways close to the periphery is generally considered to be rigid and therefore less subject to modulation. Here we demonstrate slow cholinergic modulation of information processing in a circuit traditionally seen as a fast and faithful auditory relay station. We combined electrophysiological recordings in vitro and in vivo with pharmacology and computer modeling to show that the excitability of auditory time-coding neurons is increased by the cholinergic modulation. In-vitro recordings indicate that the temporal acuity of the time-coding neurons is maintained. This study thus adds a novel component to the understanding of bottom-up dominated sensory circuitry.

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Introduction

Spherical bushy cells (SBC), the principal neurons of the anterior part of the ventral cochlear nucleus (VCN) of mammals (Brawer et al., 1974), are directly innervated by auditory nerve fibers (ANF) through giant axosomatic synapses, the endbulbs of Held (Ryugo and Fekete, 1982; Sento and Ryugo, 1989). Afferents from SBCs establish bilaterally converging inputs to both medial nuclei of the superior olivary complex (Cant and Casseday, 1986; Cant and Benson, 2003). Thus, SBC are often seen as a simple relay. A number of studies have however shown that SBC activity is not only affected by the excitatory ANF input: SBCs resting membrane potential and action potential probability are under influence of slow inhibitory synaptic inputs (Kuenzel et al., 2011, 2015; Nerlich et al., 2014b; Keine and Rübsamen, 2015) and metabotropic glutamate receptors (Chanda and Xu-Friedman, 2011; Yang and Xu-Friedman, 2015). Additionally, SBC activity is influenced by $GABA_B$ receptors (Chanda and Xu-Friedman, 2010) and the dynamics of voltage-activated conductance (Cao et al., 2007; Oertel et al., 2008). Thus it is obvious that a variety of factors influence the efficacy of information transfer from the ANF to SBC. Other neuromodulatory influences are also present, such as from the cholinergic system, but these have been less extensively studied. About three-quarters of cholinergic axons in the VCN are collaterals of the olivo-cochlear bundle fibers (OCB) originating in the superior olivary complex and thus provide stimulus-driven top-down modulation (Horváth et al., 2000; Guinan, 2006; Kishan et al., 2011). The remaining cholinergic fibers originate in the pontomesencephalic tegmentum (Mellott et al., 2011) and thus carry only indirectly stimulus-driven information. The presence of both nicotinic (nAChR) and muscarinic (mAChR) acetylcholine (ACh) receptors has been shown in VCN on a histological level (Happe and Morley, 1998; Yao and Godfrey, 1999a, 1999b; Morley and Happe, 2000; Morley, 2005; Motts et al., 2008; Hamada et al., 2010; Mellott et al., 2011; Schofield et al., 2011). Accordingly, acetylcholine can alter spike rates of neurons in the VCN (Caspary et al., 1983) and excitatory effects of ACh was shown for T-stellate cells of the

VCN (Oertel and Fujino, 2001; Fujino and Oertel, 2001; Bal et al., 2010). However, recordings from VCN neurons while simultaneously stimulating the OCB showed mixed excitatory and inhibitory effects (Mulders et al., 2002, 2003, 2009). Thus, the mechanism and functional impact of cholinergic modulation of VCN neurons, especially concerning the precisely time-coding SBC, is not yet fully resolved. This would, however, be of great interest since adaptive modulation of temporally precise information processing at the initial stages of the pathway of sound localization could have a large impact on auditory behavior. The present study thus uses both in-vitro and in-vivo electrophysiology complemented by histology and in-silico experiments to scrutinize the mechanisms through which ACh affects excitability of SBC in the gerbil VCN.

Material & Methods

The in-vitro experiments were conducted in the laboratories of the Institute for Biology 2 at RWTH Aachen University, Germany. All experiments were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by local state authorities (North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Recklinghausen, Germany).

Slice preparation

Mongolian gerbils (*Meriones unguiculatus*) aged P 14-25 of either sex were deeply anesthetized with isoflurane, decapitated and the brain quickly dissected in ice-cold cutting buffer containing (in mM): 215 sucrose, 10 glucose, 2.5 KCl, 4 Mg_2Cl 0.1 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 3 $C_6H_{12}O_6$ (myo-inositol), 2 $C_3H_3NaO_3$ (sodium pyruvate), 0.5 $C_6H_8O_6$ (L-ascorbic acid), bubbled with 95% O_2 and 5% CO_2 to a pH of 7.4 (308 mOsm). Coronal or parasagittal slices (150-250 µm) containing the rostral AVCN were cut with a vibrating microtome (VT1200S, Leica Biosystems, Nussloch, Germany). The slices were transferred into a holding chamber filled with ACSF containing (in mM): 125 NaCl, 2.5 KCl, 1 Mg_2Cl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 3 $C_6H_{12}O_6$ (myo-inositol), 2 $C_3H_3NaO_3$ (sodium pyruvate), 0.5 $C_6H_8O_6$ (l ascorbic acid), bubbled with 95% O_2 and 5% CO_2 to a pH of 7.4 (314 mOsm). Slices were incubated at room temperature for at least 30-45 minutes before recordings.

Electrophysiology

Slices were placed in a recording chamber (perfused with ACSF at 60-100 ml/h) under a fixed-stage microscope with IR-DIC and fluorescent imaging (Nikon Eclipse FN-1 microscope equipped with DS-Qi1MC camera and DC-U3 camera controller, Nikon Instruments, Japan). SBCs were patched under visual control with a HEKA EPC10 USB double patch clamp amplifier, controlled with HEKA

PATCHMASTER software (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany). Recordings were low-pass filtered at 2.9 kHz and sampled at 50 kHz. Data were analyzed with custom written software in MATLAB (The Mathworks GmbH, Ismaning, Germany). All data are presented without correcting the junction potential, which was estimated to be -11 mV. To block the GABAergic/glycinergic inhibitory inputs on SBCs, recordings were performed in the presence of 1 µM strychnine (Sigma Aldrich Germany), 10 µM gabazine (Abcam UK) and 2 µM CGP-55845 (Bio-Techne/Tocris UK). Recording pipettes were pulled from 0.86 mm/1.5 mm (inner/outer diameter) borosilicate glass filament electrodes (Science Products GmbH, Hofheim, Germany) with a horizontal Zeitz-DMZ-Universal-Puller (Zeitz Instruments, Martinsried, Germany) to have a resistance of 4-5 MΩ when filled with the recording solution. Pipettes were filled directly before recordings with patch solution containing (in mM): 100 K-gluconate, 40 KCl, 0.1 $CaCl_2$, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, 0.4 GTP, 0.1 Alexa-488 hydrazide (Thermo Fischer Scientific), 3 mg/ml biocytin (Thermo Fischer Scientific) and adjusted to a pH of 7.2 with 1 M KOH (280 mOsm). The pharmacological agents and concentrations used to isolate the cholinergic effects were: 50 µM d-tubocurarine (D-TC, Sigma Aldrich Germany), 20 nM methyllycaconitine (MLA, Sigma Aldrich Germany), 2 µM atropine, (AT, Sigma Aldrich Germany) and 100 nM tolterodine (Tol, Biotechne/Tocris UK). Recordings shown in figures 1, 3 and 5 were performed at room temperature (~23°C). To test for temperature effects, additional recordings (Fig. 4) were performed at physiological temperature (37°C), using a custom-built heated slice chamber. In these experiment, the changes of the resting membrane potential and membrane resistance upon wash-in of pharmacological agents was monitored for at least 20 minutes. This was achieved by calculating the linear fits to subthreshold current-voltage relations measured every 30 seconds (hyperpolarizing and depolarizing current steps of -100 pA to +100 pA amplitude were injected).

Pharmacological puff application and synaptic stimulation

For cholinergic stimulation we used carbachol (carbamylcholine chloride, Sigma Aldrich Germany), a stable acetylcholine analog. Carbachol was diluted in ACSF to a final concentration of 500 µM and filled into glass electrodes with a 3-4 µm tip opening. Carbachol was applied unto the patched SBCs with a 2 ms pressure-puff through a Picospritzer 2 (General Valve Corporation/Parker Hannifin Precision Fluid Systems, Hollis, USA) at 6-8 psi, yielding a restricted area of effect and a short duration of delivery of <100 ms. Area and duration of the carbachol puff application were visually adjusted with the aid of fluorescent dye (Alexa-Fluor 488, Sigma Aldrich Germany) in the pipet solution. This method has been successfully used for precisely targeted pressure application (Ko et al., 2016), although some spillover of low concentrations of the compound to neighboring cells can not be excluded. The Picospritzer was triggered by a pre-programmed recording routine in the HEKA PATCHMASTER software. This routine consisted of 10 recording sweeps, each one with a carbachol puff at the beginning, followed by a 10-second recording span, adding up to 100 seconds of recording. This allowed measuring immediate effects as well as long lasting effects on the SBC. Weighted time-constants of the decay of the cholinergic responses were calculated by fitting double-exponential functions. The weighted time constant was then calculated as $\tau_w = (A_{fast} \cdot \tau_{fast} + A_{slow} \cdot \tau_{slow})/(A_{fast} + A_{slow})$, where A_{fast} and A_{slow} are amplitudes at t=0 and τ_{fast} and τ_{slow} are the fast and slow time constants, respectively. Recordings were considered for further analysis if the fitting algorithm achieved a goodness of fit (r^2) > 0.8. This criterion resulted in omitting ten cells that showed depolarization or inward currents of unclear waveform. For synaptic stimulation, a 75 µm bipolar tungsten electrode (Micro Probe Inc., Gaithersburg, USA) of 1.5 MΩ impedance was placed in the auditory nerve root. Auditory nerve fibers were electrically stimulated with monopolar pulses generated by an Iso-Flex stimulus isolator (A.M.P.I., Jerusalem, Israel), triggered by the PATCHMASTER software. A minimal stimulation protocol was used to activate single endbulb of Held inputs. Minimal stimulus intensities were determined for each SBC (range: 5–40 V) and all further

experiments were performed at 110% threshold intensity. Sets of in-vivo like (IVL) stimulus protocols were generated offline by using the spike-time output of an auditory periphery model (Zilany et al., 2014) as stimulus arrival times (high-spontaneous rate, cat ANF). One IVL sweep consisted of 200 ms spontaneous activity and 200 ms sound driven activity (250 Hz, 40 dB SPL). A complete experiment consisted of 25 sweeps representing statistically independent draws of spike trains from the model. Sets of stimuli were identical for controls and treatment conditions. This allowed for the comparison of the mean spike probability, mean spike jitter and the in-vitro vector-strength of the cell. Pulse trains were applied by the PATCHMASTER software with 3 second intervals between the individual sweeps. To calculate the spike probability of the patched SBC, only the ratio of successful synaptic events was used, i.e. EPSPs and APs. Complete failures, i.e. lack of a detectable event after the stimulus possibly due to subthreshold stimulation, were omitted from the analysis.

Fluorescence immunohistochemistry

Gerbils aged P 18 to P 31 were sacrificed with an overdose of >150 mg/kg body weight Ketamine (Ceva Tiergesundheit GmbH, Düsseldorf, Germany) and then transcardially perfused with ice-cold phosphate buffer (PB) and 4% paraformaldehyde (PFA) in PB. The brain was removed and fixed by immersion in 4% PFA in PB overnight. After fixation, the brains were successively transferred into 10% and 30% sucrose solution for cryoprotection. Brains were embedded in Tissue-Tek (Sakura Finetek, AJ Alphen aan den Rijn, The Netherlands) and 30 µm sections were cut either the in coronal or sagittal plane on a cryotome (CM3050S, Leica Biosystems, Wetzlar, Germany). The sections were collected on gelatinized slides. Double immunohistochemical staining were performed as follows: The sections were rinsed with phosphate buffered saline (PBS) and incubated with blocking solution (4% normal horse serum (NHS), 0.4% Triton X-100, 1% bovine serum-albumin (BSA) in phosphate buffered saline (PBS)) for 3 hours at room temperature. Next, the sections were incubated with primary antibody solution (1% NHS, 0.3% Triton X-100, 1% BSA in PBS) for 24 hours at 4°C. After

rinsing with washing solution (0.02% Triton X-100, 0.25% BSA in PBS), sections were incubated with secondary antibody solution (0.02% Triton X-100, 1% BSA in PBS). The sections were rinsed with 0.25% BSA in PBS followed by a nuclear staining with 4`-6-diamidin-2-phenylindol-dye (DAPI). Finally, the sections were coverslipped with Fluoprep (bioMérieux, Basingstoke, UK), sealed against exsiccation and stored in dark at 4 °C until analysis with a laser-scanning confocal microscope (TCS SP2, Leica microsystems, Wetzlar, Germany). The concentrations, catalog-, and lot numbers (if available) of the antibodies and conjugates used were: Anti-calretinin (2 µg/ml, Merck Millipore, Darmstadt, Deutschland, Cat. No. AB1550, Lot No. 2430339), anti-vesicular acetylcholine transporter (VAChT) (3 µg/ml, Synaptic Systems, Goettingen, Germany, Cat. No. 139103), and anti-choline acetyltransferase (ChAT) (5 µg/ml, Merck Millipore, Darmstadt, Germany, Cat. No. AB143, Lot No. 2167141). All three primary antibodies are listed in the JCN antibody database (http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1096-9861/homepage/jcn_antibody_database.htm) as validated for specificity. The matching secondary antibodies were Alexa-Fluor 488 and Alexa-Fluor 546 (diluted 1:500, Thermo Fisher Scientific, Darmstadt, Germany, Cat. No. A11055 / A10040, Lot No. 1627966 / 1640319). The Alexa-555 conjugated α-Bungarotoxin (BTX) (2 µg/ml, Thermo Fischer Scientific, Cat. No. B35451) was added to the secondary antibody solution.

Biocytin-streptavidin labeling of patched SBCs

To identify the patched cells post-hoc as SBCs, the recorded cells were filled with biocytin (Thermo Fisher Scientific, Darmstadt, Germany) added to the patch pipette solution. Afterwards, the slices were fixed in 4% PFA in PB overnight, then washed 6 times in PBS for 5 minutes each, followed by 6 washing steps with 0.3% Triton X-100 in PBS for 5 minutes each. The slices were then incubated with streptavidin solution (0.1%Triton X-100, 1% BSA in PBS, 1:800 Alexa Fluor® dye streptavidin

conjugates, Thermo Fisher Scientific, Darmstadt, Germany, Cat. No. S11223) for 2.5 hours at room temperature followed by 6x 5min washes with 0.3% Triton X-100 in Tris buffered saline (TBS) followed by 3x washes with TBS only. Additionally, a nuclear staining with 4`-6-diamidin-2-phenylindol-dye (DAPI) was performed. The slices were collected on coverslips (24x60 mm) which were pasted up with a 15 x 15 mm SecureSeal Adhehsive Sheet (Grace Bio-Labs, Bend, Oregon) 240 µm in thickness and covered with a drop of Fluoprep (bioMérieux, Basingstoke, UK). The marked cells were then analyzed with a laser-scanning confocal microscope (TCS SP2, Leica microsystems, Wetzlar, Germany).

In-vivo recordings

All in-vivo experiments were performed at the Neurobiology Laboratories at the Institute of Biology of the University of Leipzig, approved by the Saxonian District Government, Leipzig (TVV 06/09), and conducted according to the European Communities Council Directive (86/609/EEC). 18 Mongolian gerbils aged between 22 and 38 days were used. Prior to the experiment, animals were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (140 µg/g body weight, Ketamin-Ratiopharm; Ratiopharm) and xylazine hydrochloride (3 µg/g body weight, Rompun; Bayer). During the experiment, subcutaneous injections of about one-third of the initial dose were applied hourly to keep the animal in an areflexic state, indicated by the absence of the limb-withdrawal reflex. Pre-experimental surgery was performed as described elsewhere (Keine and Rübsamen, 2015). In brief, the animal's skull was exposed, and two holes were drilled: the first 1.8-2.0 mm caudal from the lambda point for the reference electrode and the second 1.5 mm lateral to the former for the recordings electrode. The animal was fixed in a prone position using a brass headpost glued to the animal's skull. Glass micropipettes (GB200F-10, Science Products) were filled with 3 M KCl (impedance 5-8 MΩ) and lowered into the rostral AVCN. For iontophoretic application,

three-barrel piggyback electrodes (Havey and Caspary, 1980; Dehmel et al., 2010) were mounted to the recording electrode and filled with glycine (Sigma-Aldrich, 100 mM in ACSF, pH 6, positive control) and carbachol hydrochloride (Sigma-Aldrich, 5-500 mM in ACSF, pH 6), respectively. A backing current for each barrel was used (-15 nA) and the third barrel filled with 1 M sodium acetate served for current balancing. In a subset of experiments, four-barrel electrodes were used and the fourth barrel filled with ACSF, omitting glycine and carbachol, served as negative control. Drugs were applied iontophoretically (EPMS 07; npi electronic) with increasing current steps (+0 to +100 nA; automatic capacitance compensation was applied), while spontaneous activity of the neuron was monitored. The rostral pole of the AVCN was targeted considering the tonotopic organization of the nucleus (Kopp-Scheinpflug et al., 2002; Dehmel et al., 2010) and SBCs were identified by their characteristic complex waveform (Pfeiffer, 1966; Winter and Palmer, 1990; Englitz et al., 2009; Kuenzel et al., 2011; Keine and Rübsamen, 2015). Extracellular voltage signals were digitized at a sampling rate of 97.7 kHz (24 bit, RP2.1; Tucker-Davis Technologies), band-pass filtered between 50 Hz and 5 kHz, stored and analyzed using custom-written Matlab software (version 8.5, The MathWorks, Natwick, USA).

Spherical bushy cell model

Simulations were performed as previously published (Kuenzel et al., 2011, 2015; Nerlich et al., 2014b) with NEURON (Hines and Carnevale, 1997, 2000; Hines et al., 2009) using custom-written software in Python 2.7 under Linux x86_64 for simulation control and analysis. Properties of ionic conductances and membrane biophysical properties of the SBC model were set to match previously published models (Rothman and Manis, 2003), except the sodium channel model we used, which was published earlier (Rothman et al., 1993). Briefly, a somatic compartment, an axon first segment containing all voltage-activated sodium conductance and a stretch of passive axon were included in

the model. Reversal potential for the leak conductance (gLeak total: 14.5 nS) was set to -65 mV. Voltage-activated ion channel conductances were Na_v 1000 nS, LVA-K 200 nS, I_h 40 nS (E_{rev} -43 mV) and HVA-K 175 nS. Basic somatic parameters of the model SBC, therefore, resulted in a total membrane capacitance of 20.1 pF, a total input resistance (at rest) of 69.2 MΩ, and a resting membrane potential of -65.1 mV. All simulations were run at a temporal resolution of 10 µs. A model of the endbulb of Held giant terminal and an inhibitory synaptic input (representing the sum of GABA/glycinergic inputs to the SBC) were connected to the somatic compartment. Synapses were modeled as conductance point sources (Excitation = 55 nS, E_{rev} = 0 mV; Inhibition: 24 nS, E_{rev} = -75 mV), synaptic dynamics were included as a Gaussian distribution of EPSG amplitudes for the endbulb of Held (55 ± 9 nS) (Nerlich et al., 2014b) and as complex rate-dependent plasticity for the inhibitory input (Nerlich et al., 2014a, 2014b). For some simulations, inhibitory conductance was set to 0 nS and excitatory conductance was reduced accordingly to match again the failure rates of endbulb of Held synapses observed in vivo. Conductance waveforms for the synaptic mechanisms were generated by convolving EPSG template waveforms with the spike arrival times of the respective inputs at the temporal resolution of the simulation. Inhibitory inputs were delayed by 1 ms compared to excitatory inputs. The nicotinic synaptic input to SBC was simulated by an additional conductance point source connected to the soma of the SBC model. Parameters of the nicotinic mechanism were extracted from the in-vitro recordings: rise-time 83 ms, peak amplitude 2 nS, decay time constant 461 ms, E_{rev} 0 mV (data for E_{rev} not shown). An additional onset delay of 10 ms compared to the onset of the auditory nerve inputs was applied to the nicotinic input, as reported for the latency of the olivocochlear bundle effect (Brown et al., 2003). Simulated sound responses were paired with the nicotinic input. The muscarinic modulatory effect on the resting membrane potential was simulated by de- or hyperpolarizing the reversal potential of the leak conductance to -55 mV (simulating the +ACh condition) or -75 mV (simulating the -ACh condition with atropine). Note that no onset or offset dynamic of the modulatory effect was included in the

model, thus all simulations of modulatory effects focus on reviewing the impact of the fully developed phenomenon on the SBC function.

Spike arrival times for the primary inputs driving the excitatory and inhibitory synaptic mechanisms were generated with an inner ear model (Zilany et al., 2014) implemented in the Python module 'cochlea' (Rudnicki, M. and Hemmert, W., 2014, Cochlea: inner ear models in Python, https://github.com/mrkrd/cochlea, version 1.2). Pure tones of 500 ms duration (including 2 ms $\cos^2$ ramps) were presented repetitively (100x) at various sound-pressure levels (-10 to 75 dB SPL) and frequencies (125 to 4000 Hz) to the inner ear model. A set of AN output spikes for high spontaneous rate AN fibers ('cat' parameters) were generated at a temporal resolution of 10 µs. Low frequency AN fibers (CF=1200 Hz) were routinely simulated. For the generation of PSTH, higher frequency AN fibers (CF=3 kHz and tones >3000 kHz) were simulated to prevent effects of phase-locking. The same set of auditory nerve responses was used for the different experimental conditions to facilitate comparison of the cholinergic effects. SBC spikes and failures were detected and waveforms of events were analyzed as previously published (Kuenzel et al., 2011, 2015). Using the peak of the SBC action potential as spike-time, we constructed rate-level functions, peri-stimulus time histogram and cycle-histograms. Temporal precision was quantified with the vector strength of phase-locking (Goldberg and Brown, 1969), a Rayleigh criterion of $p<0.001$ was used.

All data are presented as mean ± standard error of the mean, unless indicated otherwise. Statistical significance of the influence of the different pharmacological treatment conditions was tested with the Kruskal-Wallis test with Bonferroni-corrected post hoc testing, except for the data set depicted in Fig. 5, where paired t-tests were used.

Results

In vitro recordings in acute parasagittal slices were performed at the rostral pole of the gerbil AVCN (P14-P25) where the low-frequency SBCs are located (Oertel, 1983; Wu and Oertel, 1984). During the experiment, SBCs were identified by their large round somata visualized by the IR-DIC optic of the microscope and additionally verified posthoc in a subset of recorded cells (n=26) by images showing the somata and the typical bush-like dendritic morphology in biocytin-streptavidin staining (Fig. 1A). In addition, identification of SBC was augmented by electrophysiological features: (i) during suprathreshold depolarizing current injections they generate only one (or two) action potentials and (ii) the membrane voltage shows a prominent voltage sag upon steady hyperpolarizing current injection (Fig. 1B). Furthermore, synaptic stimulation of the AN elicited large, depressing EPSC in SBCs (Fig. 1C). The basic biophysical properties (R_m = 95 ± 6 MΩ, C_m = 24.4 ± 2.3 pF, n=78) were consistent with previously reported data (Oertel, 1983; Schwarz and Puil, 1997). Average resting membrane potentials of the SBC were -57.8 ± 0.9 mV (n=78; uncorrected).

Carbachol elicits a transient nicotinic inward current in spherical bushy cells

To assess the cholinergic responses of SBC, carbachol was puff-applied to the cell in current-clamp recordings. Fifty-five percent of tested SBCs (23 of 42) exhibited some form of transient depolarization. We quantified this in cells where a double-exponential fit of the decay waveform yielded a good match with the actual measurements (r^2>0.8; see material & methods) and found on average a depolarization of 3.7 ± 0.5 mV (Kruskal-Wallis $p<0.05$, df=29, χ^2=20.05, n=13; post-hoc $p<0.01$ vs ACSF control) relaxing back to the resting potential with a time constant of 398±298 ms (Fig. 1Di). Carbachol puffs in the presence of d-tubocurarine (D-TC), a general blocker of nicotinic receptors, yielded no depolarization (0.1± 0.1 mV, n=8, $p<0.01$ vs. carbachol condition) indicating the specificity of the response (Fig. 1Dii). To further explore the composition of the nicotinic receptors

present on SBCs, we used methyllycaconitine (MLA), a blocker specific to α7 subunit containing nAChR (Fig. 1Diii). Also in the presence of MLA, the carbachol triggered depolarization was completely abolished (0 mV, n=4, $p<0.01$ vs. carbachol condition). Puff-application of ACSF alone yielded no response (Fig. 1Div) (0 mV, n=5, $p<0.001$ vs. carbachol condition). Population data for the depolarization are shown in Fig. 1E.

In voltage-clamp recordings at a holding potential of -60 mV, puff application of carbachol unto SBCs elicited inward currents (-73.5 ± 22.7 pA, Kruskal-Wallis $p<0.05$, df=33, $\chi^2=25.94$, n=19; post-hoc $p<0.001$ vs. ACSF control, Fig. 1Fi), which relaxed back to rest with a time constant of 461 ± 117 ms and had a reversal potential of >0 mV (data not shown). The differences in decay time-constants derived from the two recording methods are not statistically significant. The peak inward current corresponds to a peak conductance of 1.2 ± 0.4 nS. The wash-in of D-TC completely abolished the inward current (-1.7 ± 0.6 pA, n=7, $p<0.001$, Fig. 1Fii), as did wash-in of MLA (-0.2 ± 0.2 pA, n=4, $p<0.001$, Fig. 1Fiii). The specificity of the response was again shown by the fact that puffing ASCF alone yielded no inward current (-0.5 ± 0.3 pA, n=4, $p<0.01$, Fig. 1Fiv). The population data for the transient inward current are shown in Fig. 1G. Combined, these results show that, similar to T-stellate neurons in the AVCN (Fujino and Oertel, 2001), SBC express functional nAChR that mediated depolarizing inward currents with slow temporal characteristics when compared to the excitatory glutamatergic auditory nerve fiber input (Isaacson and Walmsley, 1996; Gardner et al., 1999; Xie and Manis, 2013). The complete block of response by MLA suggests that, unlike stellate neurons (Fujino and Oertel, 2001), SBC exclusively express an α7 containing subtype of nAChR.

Differential distribution and subcellular localization of cholinergic transmission in the Gerbil's VCN

In order to confirm the presence of cholinergic innervation in the CN of the gerbil and to determine the distribution of putative cholinergic synapses within the nucleus, immunohistochemical staining

was performed in sagittal and frontal slices of the CN in brains prepared from P 18 - 31 gerbils. Fluorescent labeling of calretinin (CR, green in Fig. 2 Ai, Bii, C, F) and DAPI (blue in Fig. 2Aii, Bi & E) was used as reference to visualize the different subnuclei of the CN and to identify the SBCs by the large, CR-positive somatic endbulb terminals.

To visualize the en-route cholinergic fibers in the CN we used an antibody against choline-acetyltransferase (ChAT). To show presynaptic structures of the cholinergic system we employed an antibody against the vesicular acetylcholine transporter (VAChT). Staining against ChAT revealed punctuated immunosignals in the neuropil throughout the whole CN (Fig 2Aii), with a higher signal intensity in the PVCN than in the rest of the CN (Fig. 2Aii, arrowhead). Staining arranged in stripes was also seen in the auditory nerve (Fig. 2Aii, arrow), possibly representing cholinergic axons innervating the cochlear root neurons (Gómez-Nieto et al., 2013) or cholinergic collaterals entering the CN via this route. However, we cannot fully explain the staining pattern seen in the nerve root at this moment. VAChT-positive immunosignals were found throughout the entire VCN (Fig. 2Bi), and the VAChT antibody also reliably labeled cholinergic terminals (Fig. 2Bii, magnification of the area marked by the white box in Bi). The VAChT staining revealed both VAChT-positive fibers taking a rostro-caudal course (arrow) and *en passant* swellings (arrowheads), showing presynaptic cholinergic structures in the immediate vicinity of SBCs. These structures were also found in the PVCN (Fig. 2Bi, arrows). In frontal sections of the AVCN the VAChT positive fibers (arrow) were less numerous (Fig. 2C), suggesting a mostly rostro-caudal course of these fibers. VAChT positive immunosignals were also regularly found in the granular cell layer of the CN (Fig. 2D, dotted lines; GCD), with the signal strength even surpassing that in the SBC domain of the AVCN. This is in agreement with previous reports of cholinergic innervation of the CN-GCD (Brown et al., 1988) together with inputs from non-auditory sources (Gómez-Nieto and Rubio, 2009; Zeng et al., 2012). VAChT positive immunosignals were also observed in the area of the auditory nerve root (ANR, Fig. 2D, arrow) consistent with previous studies showing cholinergic innervation of ANR neurons in rats (Gómez-Nieto et al., 2013).

Postsynaptic sites associated with cholinergic transmission were identified by fluorescently conjugated α-Bungarotoxin (α-BTX) which specifically binds to nAChR. An overview of the VCN shows strong staining of AVCN and somewhat weaker staining of the PVCN (Fig. 2E). No immunosignals were found in the auditory nerve region which can be regarded as additional confirmation for the specificity of the α-BTX labeling. In high magnification confocal images of the AVCN (Fig. 2F, frontal section), the α-BTX immunosignals appeared mostly located in the neuropil surrounding SBCs but rarely in close association with SBC somata. These signal locations indicate a potential dendritic expression of nAChR in SBC. Especially in low magnification the "postsynaptic" α-BTX labeling appears stronger and more diffuse than the "presynaptic" ChAT / VAChT signals, which might indicate focal vs. widely distributed expression of the respective target proteins and suggests volume transmission. Taken together, our histological findings agree with the suggestion that the cholinergic inputs enter the rostral AVCN in a caudo-rostral direction and tend to target the dendritic region of SBC rather than their somata.

Muscarinic Acetylcholine receptors mediate a long lasting RMP modulation

In addition to the transient depolarization through α7 nAChRs shown above, longer lasting depolarizations were seen in 76% of the SBCs (32 of 42 cells) upon repeated application of carbachol. Presenting 10 carbachol puffs over a period of 100 seconds caused an average depolarization of 2.6 ± 0.1 mV (Kruskal-Wallis $p<0.05$, $n=32$, $df=56$, $\chi^2=43.39$; post-hoc $p<0.001$ vs ACSF control; Fig. 3A & G "ACh"). After the end of the carbachol application the SBCs' resting membrane potential continued to drift to even more depolarized values. The mean maximum shifts amounted to 5.6 ± 0.2 mV (reached after 11 ± 1 minutes; $p<0.001$ vs ACSF control, $p<0.01$ vs ACh 100sec, $n=13$; Fig. 3B & G "ACh max"). Thereafter the resting membrane potential recovered with a similar temporal profile to the initial values (Fig 3B).

To test whether this depolarization is due to the activation of muscarinic acetylcholine receptors (mAChR), carbachol stimulation was performed following a wash-in of atropine (2 µM), a general mAChR blocker, and of tolterodine (100 nM), a synthetic high-affinity mAChR blocker. Surprisingly, both blockers caused a reversible hyperpolarization of SBCs, as seen from the average peak effects: Atropine by -5.3 ± 0.2 mV ($p<0.001$ vs ACSF control, n=6; Fig. 3G) and tolterodine by -12.1 ± 1.6 mV ($p<0.001$ vs ACSF control, n=3; Fig. 3C & G). This suggests a tonic activation of muscarinic receptors contributing to the SBCs RMP in vitro. After a new stable hyperpolarized RMP was reached following either of the two pretreatments, the puff-application of carbachol caused no significant SBC depolarization anymore (-0.1 ± 0.1 mV, $p<0.001$ vs ACh max, n=5, Fig. 3E & G). When ACSF was puffed as a control, no depolarization was noticeable, neither after 100 seconds (-0.1 ± 0mV, n=3) nor after 20 minutes (-0.2 ± 0.4 mV, n=3, Fig. 3F & G). However, SBC still showed the nicotinic depolarization in the presence of atropine (2.6 ± 1 mV, n=3, data not shown). Furthermore, the muscarinic depolarization was visible in the presence of D-TC or MLA (2.3 ± 0.3 mV after 100 seconds, n=6, data not shown).

The majority of our in-vitro experiments was performed at room temperature (~23°C). To rule out any adverse effect of the low temperature, especially on G-protein mediated processes, we repeated the wash-in experiments with tolterodine at physiological temperature (37°C, Figure 4). Here, we monitored the change of SBC resting membrane potential and membrane resistance before, during and after wash-in of tolterodine. Similar to the experiments at room temperature, we observed a hyperpolarization of the RMP of -9.9 ± 3.8 mV (average of 10 minutes of wash-in; n=6; Fig. 4A & B). The average RMP during wash-in of tolterodine was significantly different from average RMP before and after wash-in (Kruskal-Wallis $p<0.05$, df=17, $\chi^2=9.1$; post-hoc U-test $p<0.05$ “Tol” vs. “Ctr” and “Tol” vs. “Rec”, p=0.94 “Ctr”. vs. “Rec”). Surprisingly, we did not observe a consistent change of R_m during wash-in of tolterodine (Fig. 4C), possibly due to the comparably large variability given the low absolute R_m of the SBC. Although R_m slightly diminished upon wash-in (Control 69 ± 9 MΩ,

Tolterodine 62 ± 14 MΩ), this difference was not statistically significant (Kruskal-Wallis p=0.31, df=17, χ^2=2.3) and furthermore did not recover (Recovery 58 ± 12 MΩ) after switching the perfusion back to ACSF.

Thus, in addition to (and independently of) the transient depolarization mediated by nicotinic AChR, SBCs RMP is slowly shifted to depolarized values by carbachol through the action of muscarinic AChR. This mechanism is independent of temperature and appears tonically active in SBC. Thus, mAChR critically contribute to determining the RMP of the SBC.

The cholinergic RMP depolarization increases SBC's spiking probability in vitro

Next, we tested whether the carbachol induced depolarization affects the probability of AP generation and also the temporal precision of the SBC spiking. For this, minimal electrical stimulation of ANF inputs to SBC was paired with the puff application of carbachol. For the synaptic stimulation, "in-vivo-like" (IVL) temporal stimulation patterns were employed mimicking spontaneous and sound driven activity by high spontaneous-rate ANF (Fig. 5). This resulted in SBC spike probability >20%. If SBC had a spiking probability of 0% (i.e. showed only EPSPs) or 100% (only action potentials), they were not included in this analysis because of the limited dynamic range on either end. If a stable synaptic stimulation was achieved and the control spike probability was established, we applied our carbachol stimulation paradigm (10 puffs, 0.1Hz). When the cells showed a clear cholinergic reactivity, we repeated the synaptic stimulation with an unchanged stimulus intensity. Under carbachol influence, the cells showed a significant increase in spiking probability (paired t-test p<0.01, n=5; Fig. 5Cii & Dii "ACh"). To test if the increased spike probability was directly caused by the carbachol-mediated RMP elevation, we paired the synaptic stimulation with an RMP elevated by 5.5 mV through current injection in a different set of cells. In these experiments, we also observed a significant increase in spike probability (paired t-test p<0.01, n=5). It increased to a similar extend as

it did for the cholinergic modulation (Fig. 5Ciii & Di "I_{Inj}"), although a direct statistical comparison between the carbachol application and current-injection was not performed. Because the SBC showed a large variability in their spike probability under control conditions, we calculated the mean relative increase for all paired recordings, showing that the spike probability significantly increased more than twofold for both treatment conditions (ACh=2.2 ± 0.9 fold increase, RMP=2.3 ± 0.4 fold increase, paired t-tests $p<0.01$, Fig. 5Dii).

Because the temporal precision is one of the prime characteristics of binaurally converging input to the medial superior olive (MSO), we explored in vitro how the carbachol-induced increase in SBC AP probability influences the timing of APs. The temporal AP jitter, quantified as the average standard deviation of the AP latency, decreased from 0.32 ms (control conditions, n=5 for each condition) to 0.17 ms in carbachol treated cells (n=5) and 0.17 ms (n=5) in cells which were depolarized through current injection, however did not reach statistical significance in both cases (data not shown). For the carbachol treated cells, mean vector strength (VS) was 0.67±0.04 (n=5) compared to 0.65±0.03 (n=5) under control conditions (Fig 5Ei & Eii). When SBCs were depolarized through current injection the initial VS values were 0.66±0.04 (n=5) and increased to 0.69±0.05 (n=5) for elevated membrane potentials (Fig 5Ei & Eii). Due to the paired paradigm we did not test whether VS differed for carbachol treatment vs. current injection. The input VS of the IVL stimuli was 0.7 in all experiments. On average, the mean absolute change in vector strength was minimally positive and furthermore statistically not significant. Taken together, we found the vector strength to remain stable in cells subjected to carbachol-induced depolarization or direct depolarization through current injection (Fig. 5Ei).

In summary, the action of mAChR increased the spike probability in vitro by bringing the SBCs closer to their firing threshold but at the same time did not interfere with the temporal precision of spike timing. These data suggest that the muscarinic component of the cholinergic modulation causes a twofold increase in well-timed output spikes without a reduction in temporal precision, i.e. the

synchronization rate of the SBC output is substantially increased by the action of the cholinergic top-down system.

Carbachol increases the spiking rate of SBC in vivo

Next we tested if the increase of spike-probability caused by the influence of cholinergic inputs on the RMP can also be observed in the intact brain of anesthetized gerbils. To this end single-unit recordings were acquired from a total of 24 identified spherical bushy neurons in the rostral AVCN of gerbils aged P 22 - 38, while iontophoretically applying carbachol (Fig. 6). When using a low concentration of 5 mM carbachol (Fig. 6A), none of the units (n=4) tested responded with a change in spontaneous firing rate. The spontaneous rate profile over the 30 s of iontophoresis was comparable to that of the control ACSF application (Fig. 6H), and average rate was not statistically different from negative controls (p=0.38, 105 ± 4% for 5 mM carbachol vs. 101 ± 10% for ACSF control). Iontophoretic application of glycine, however, transiently reduced the spontaneous firing rate (asterisk in Fig. 6A) in the same units, indicating responsiveness of the spontaneous firing rate in these units to iontophoresis of pharmacological agents. No significant changes (P=0.63) in SBC spontaneous firing occurred upon iontophoresis with ACSF (n=7; average response to ACSF is shown in Fig. 6G). Higher concentrations of iontophoretically applied carbachol (100 mM, n=9; 200 mM, n=7; 500 mM, n=4) resulted in a significant deviation of the spontaneous firing rate. Iontophoresis of 100 mM resulted in a slow and delayed rise in spontaneous action potential firing (on average to a maximum of 200% after 30 s; mean rate 146 ± 22%) that gradually returned to baseline after the end of the iontophoretic currents (Fig. 6B). Higher concentrations of carbachol (Fig. 6C-F) caused on average a more rapid increase in spontaneous SBC firing (Fig. 6H) and a higher average spontaneous spike-rate during drug application (Fig. 6I). At 200 mM, a maximal increase to 160% after 12 s was observed (mean rate 159 ± 24%), at 500 mM spontaneous spike rate was increased to a maximum of

250% after 21 s (mean rate 175 ± 68%). However, in a number of cases a reduction of spontaneous firing rates followed the initial intense increase of spontaneous spiking (Fig. 6D & F) during the ongoing iontophoretic drug application. This resulted in a phasic-like response to the carbachol iontophoresis and was quite common in the two highest concentrations, as can be seen from the average response profiles per concentration (Fig. 6H), but never occurred with 100 mM carbachol. On the other hand, even at 500 mM carbachol the reduction of spike rate was absent in one unit (Fig. 6E). In all cases this effect could be distinguished from a reduction of measured spike rates caused by pure technical reasons (e.g. "losing the unit", see end of recording in Fig. 6C for example), because the reduction of spiking caused by the iontophoretic drug application recovered to the steady state spontaneous firing rate after some time or did not go below this value at all. At this moment we cannot exclude unspecific responses to the iontophoresis (e.g. effects of pH) as an explanation for the reduction of the spontaneous rate in some of the SBC upon strong carbachol iontophoresis.

The mean effect of the iontophoretic application of carbachol, averaged over all the SBC and the concentrations (n=24 SBC, n=4 concentrations) used, is shown in Figure 6G. Over all units, carbachol iontophoresis causes a significant increase in spontaneous spike rate to 148% after 20 s (mean 139 ± 7%, $p<0.01$). After the end of the iontophoretic current, the spontaneous spike rate reached baseline values after 16 s. Taken together, a significant increase of spontaneous spike rate was seen upon carbachol application to SBC in vivo. In this set of in-vivo experiments, we did not apply sound stimulation and therefore cannot yet comment on the functional impact of cholinergic modulation on temporal coding and signal processing. It is nevertheless evident that in the intact adult gerbil brain cholinergic responses of SBC cause a slow and sustained increase in resting spike probability consistent with our observations in acute brain-slice experiments.

Functional relevance of the cholinergic modulation of SBC excitability

We next assessed the potential functional impact of the cholinergic inputs to SBC in the context of the auditory sensory role of SBC using an in-silico approach. The in-silico approach was chosen a) because our in-vivo experiments lack responses to acoustic stimulation and b) to differentially analyze the interaction of sound stimulation with the nicotinic transient and the muscarinic modulatory effect. In an SBC model used in previous studies (Kuenzel et al., 2011, 2015; Nerlich et al., 2014b) we implemented the transient nicotinic input as a weak (2 nS), long-lasting (risetime=83 ms, decay time-constant=461 ms) depolarizing cation conductance (E_{rev}=0 mV). When paired with excitatory inputs driven by simulated auditory nerve inputs (Fig. 7Ai, auditory stimulus indicated by horizontal gray bar) the nicotinic post-synaptic potential (PSP) caused an increase in spike-probability (P_{AP}) in the SBC model. Since we imposed a 10 ms delay of the nicotinic event with respect to the sound response onset, the increase in P_{AP} is especially pronounced in the early ongoing phase of the sound stimulation, as can be seen in average peri-stimulus time histograms (green = control without nicotinic input, black = with additional nicotinic input Fig. 7Aii). During this segment of the response, the number of failures due to the accumulated effects of refractoriness and the impact of acoustically evoked inhibition is usually increased (Kuenzel et al., 2011; Keine and Rübsamen, 2015). The additional depolarization caused by the nicotinic event, however, sufficed to push a significant number of events back above threshold. Analyzed over many stimulus levels, this effect was more pronounced at higher response rates, i.e. higher SPL (Fig. 7Aiii). Nevertheless, also for very low SPL the nicotinic modulation did increase the signal-to-noise ratio between stimulus responses and spontaneous baseline (Fig 7Aiii inset).

Next, we simulated the fully developed muscarinic modulation as a shift of the reversal potential of the leak conductance. Given the results of the in-vitro experiments (Figs. 3-5) and our inability to extract a consistent muscarinic conductance change from our data, we deemed this a sufficiently good “first-order” approximation of the observed muscarinic effect. The simulated RMP changes

affected P_{AP} especially during higher firing rates, i.e. short inter-spike intervals. The simulated block of nicotinic RMP elevation by atropine (E_{rev}= -75 mV; Fig 7Bi, red) resulted in more failures than under control conditions (Fig. 7Bi, green). Furthermore, during the simulated carbachol condition (E_{rev}= -55mV; Fig. 7Bi, black) even fewer failures occurred. In the simulated atropine condition, a higher number of failures occurred and the effects of refractoriness at higher stimulus levels (Fig. 7Bii) were more pronounced than for the control condition (E_{rev}= -65 mV). The impact of the muscarinic modulation became most obvious during the rising slope of the rate-level function (0-20 dB, Fig. 7Bii), i.e. the dynamic response range of the unit. Similar to the nicotinic effect, the simulated carbachol condition (E_{rev}= -55 mV) resulted in a further elevation of P_{AP} compared to the control condition. In contrast to the nicotinic event, the modulatory effect did indeed increase spontaneous firing rates as well, as seen in the in vivo recordings (cf. Fig. 6; arrows in Fig. 7Bii). When we simulated a complete frequency response area for each condition (Fig. 7Biii) we saw that the general shape of a contour where the response was close to threshold (5 spikes/s elevated over the spontaneous response, dotted lines), was indistinguishable between conditions. Thus, for low stimulus levels the modulatory effect does not enhance the signal-to-noise ratio. However, the response width of the simulated atropine condition markedly deteriorated with higher response levels, as can be seen for the 50 spikes/s-elevation contour (also cf. Fig. 7Bii, solid lines). Thus, in the context of the in vitro and in silico results, a depolarized RMP caused by moderate ambient ACh levels increases excitability and extends the dynamic range of tuning of the SBC.

We next investigated whether the two nicotinic effects have an impact on spike-time precision and thus phase-coding of the SBC. Therefore, responses to a range of pure tones under the different ACh conditions were simulated. The interaction of excitatory and nicotinic inputs caused an amplitude increase in the period histograms (Fig. 7Aiv) with only very little change of preferred phase. Over all frequencies tested, the nicotinic input resulted in a small increase in vector strength, especially for frequencies >1 kHz (Fig. 7Av). Results were more pronounced for the simulated modulatory effect

(Fig. 7Biv/7Bv). Increasing the cholinergic modulation (i.e. depolarizing the RMP) resulted in markedly enhanced period histogram amplitudes, an advance of preferred phase (Fig. 7Biv) and more pronounced improvement of phase locking accuracy (Fig. 7Bv). On the other hand, reduction of cholinergic modulation (i.e. hyperpolarizing the RMP) resulted in reduced period histogram amplitudes, a phase delay (Fig. 7Biv) and impaired phase-locking (Fig 7Bv).

Taken together, in-silico experiments suggest that both nicotinic and muscarinic modulation of SBC increases the output of well-timed action potentials and that a baseline of muscarinic activation is necessary to set the working RMP and excitability of SBC.

Discussion

Here we propose a novel mechanism for tuning the RMP and spike probability of time-coding sensory neurons in the auditory brainstem of mammals through cholinergic signaling. We showed that SBCs RMP is modulated on different time scales through nicotinic and muscarinic AChRs. While the activation of α7-nAChRs transiently depolarized the SBCs with a time constant of several hundred milliseconds, the level of activation of mAChRs dynamically determined the RMP in a range of several minutes. Cholinergic modulation leads to an increased spiking probability in vitro by moving the membrane potential closer to threshold. The temporal acuity of the SBC output, however, was stable despite the increase in P_{AP}. The in vitro data were supported by in vivo single-unit recordings combined with pharmacology, where carbachol iontophoresis increased spontaneous spike activity of SBC. In agreement with the experimental results, an SBC model that included a first representation of the cholinergic effects predicted an increase in spiking responses, expanding the dynamic sound response range of the neuron and increasing the number of well-timed output action potentials. Together, our results suggest top-down control of information processing in monaural time-coding neurons in the initial stages of the auditory pathway mediated by cholinergic modulation.

The AChR composition of SBCs differs from other cell types in the CN

The existence of cholinergic signaling in the cochlear nucleus has been investigated before for T-stellate cells in the VCN (Fujino and Oertel, 2001), cartwheel and giant cells in the DCN (Pál et al., 2009; He and Wang, 2014) as well as for cells of the granular cell domain (Irie et al., 2006; Kőszeghy et al., 2012). Cholinergic signaling onto time-coding neurons like the SBC has not been systematically analyzed yet (but see: Oertel and Fujino, 2001; Bledsoe et al., 2009). We found that in SBCs only α7-subunit containing receptors are expressed. We thus conclude that SBC solely express the α7 homopentamer nAChR (Happe and Morley, 1998; Yao and Godfrey, 1999a, 1999b; Morley and Happe, 2000; Morley, 2005). Our histological data indicate dendritic localization of the AChR, a feature that has also been shown for other secondary inputs on SBC (Gómez-Nieto and Rubio, 2009).

The α7 nAChR has a high Ca^{2+} permeability (Berg and Conroy, 2002), which could explain the drop of in-vivo spontaneous spike rate after carbachol application in some of our in-vivo recordings. Unphysiologically high nicotinic activation could possibly cause Ca^{2+}-dependent modifications of potassium- or HCN-channels or activation of Ca^{2+}-dependent conductance, even though SBC show strong expression of calcium binding proteins (Bazwinsky et al., 2008). It can, however, not be ruled out that the reduction of spontaneous rate represents an entirely unspecific effect of the iontophoresis (cf. Franken et al., 2015).

The sole expression of the α7 receptor subtype is different from that in stellate cells, which express a mixture of α7 and α4β2 containing nAChRs (Fujino and Oertel, 2001). D-stellate cells and octopus cells (Oertel and Fujino, 2001; Fujino and Oertel, 2001) on the other hand show no AChRs at all. Physiological data for the DCN is restricted to muscarinic receptors, since they account for most of the cholinergic signaling in the DCN (Irie et al., 2006; Pál et al., 2009; Kőszeghy et al., 2012; He and Wang, 2014). In the DCN combinations of m1-4 mAChR expression has been reported (Kejian et al., 1994; Godfrey et al., 1998; Irie et al., 2006; Pál et al., 2009; Zhao and Tzounopoulos, 2011; He and Wang, 2014). For the AVCN, only the existence of the m2 and m3 subtypes of mAChRs could be shown (Yao and Godfrey, 1995, 1996; Yao et al., 1996; Hamada et al., 2010). Since the m3 receptor mediates postsynaptic excitation by inhibiting potassium currents (Brown, 2010; Haga, 2013), a phenomenon termed the M-current (Brown and Adams, 1980), we hypothesize that the modulation of SBC RMP is caused by m3 mAChR activation. However, since we could neither show a consistent increase nor decrease of R_m upon muscarinic activation, the exact mechanism of the muscarinic modulation of SBC remains to be investigated.

Endogenous ACh sets SBC's RMP

Pharmacological blockade of mAChRs strongly hyperpolarized SBCs. We hypothesize that already at rest, SBCs are tonically depolarized by endogenous ACh released in the vicinity of SBCs, participating in setting the RMP and the excitability of the SBC. The idea of RMP modulation by endogenous ACh

has also been proposed for fusiform and cartwheel cells of the DCN (Godfrey et al., 1998). It has also been shown that SBC's RMP is set by endogenous glutamate through mGluRs (Chanda and Xu-Friedman, 2011; Yang and Xu-Friedman, 2015). Although we could only represent the muscarinic effect in a simplified form in the model, some general conclusions can be drawn from our in-silico experiments: First, as shown by the simulated atropine / hyperpolarized condition, a baseline ACh level seems necessary to achieve a functional excitability and dynamic response range of the model SBC. Second, further cholinergic input (the simulated carbachol / depolarized condition) positively influences SBC P_{AP}, especially in conditions with numerous failures and also improves temporal precision.

Functional implications of different time constants of cholinergic modulation

Although our model of the muscarinic modulation was simplified and we did not perform combined in-vivo pharmacology and sound-stimulation, our in silico results nevertheless suggest that both nicotinic activation and full muscarinic modulation increases P_{AP} and dynamic range of SBC. Together with the increase in P_{AP} we found an increase in temporal precision in the model under both nicotinic und muscarinic modulation. The combined increase in rate and precision causes a marked increase in the number of well-timed SBC action potentials, which could suffice to improve detection and localization of tones in noise by enhancing ITD cues used for binaural unmasking further up in the auditory pathway. Also, responses of SBC to any sound-stimulus that occur temporally aligned with the nicotinic input to SBC will cause more output spikes compared to unmatched stimuli, without a deterioration of output precision. This is especially pronounced at mid to high SPL, where the additional depolarization by the nicotinic PSP helps to overcome refractoriness and the tonic inhibition (Nerlich et al., 2014b) in the ongoing segment of the sound response. Based on our results we hypothesize that the nicotinic and muscarinic receptors possibly mediate the same effect at different time scales. This also seems probable with respect to the two time scales the OCB exhibits.

Cholinergic modulation of time-coding neurons

The fast olivocochlear effect works in the range of tens of milliseconds, whereas the slow effect lasts up to minutes (Guinan, 2006). The nicotinic activation of SBCs could provide a fast but phasic reaction which is activated during the onset of the peripheral olivocochlear effect. The muscarinic activation, on the other hand, could provide a long lasting modulation that is activated during the slow and ongoing olivocochlear effect. The activation of medial fibers of the OCB reduces the gain of the cochlea and enhances the detection of tones in noise (Guinan, 2006), but at the cost of a reduced dynamic range. Neuronal processing in low-frequency spherical bushy cells, which only receive one or very few AN inputs, already imposes a reduction of dynamic range compared to AN inputs due to synaptic properties (Wang and Manis, 2008) and interaction with inhibitory inputs (Kuenzel et al., 2011; Keine and Rübsamen, 2015). The total loss of dynamic response range at the SBC output level during OCB activation could thus impair sound processing downstream of SBC. However, the increased excitability of cells in the CN caused by cholinergic innervation through collaterals of the OCB, which seem to make up the most of the cholinergic innervation in the CN (Mellott et al., 2011), serves to ameliorate the total loss of dynamic range by making SBC input and output more similar.

ACh modulates auditory processing

In the auditory system, ACh has been implicated in regulating auditory plasticity (Keuroghlian and Knudsen, 2007). In general, widespread modulation of the brain through ACh is implicated in setting the behavioral state of the animal (Lee and Dan, 2012; Aton, 2013). It has been shown that attention, motivation and vigilance of the animal, mediated largely by ACh, greatly influence sensory processing (Pepeu and Giovannini, 2004; Hasselmo and Sarter, 2011; Lee and Dan, 2012; Smucny et al., 2015), e.g. decreasing sensory detection thresholds for attended stimuli while increasing thresholds for the unattended stimuli (Aton, 2013). We hypothesize that cholinergic signaling in the AVCN of the gerbil is partially under top-down control from brain areas that are not immediately within the auditory pathway. It has been shown that apart from OCB input, cholinergic projections from the

pontomesencephalic tegmentum enter the CN (Mellott et al., 2011). This nucleus regulates arousal and vigilance and is under direct cortical control. Also, the OCB signaling is under additional cortical control (Doucet et al., 2002). It is intriguing to speculate that through ACh SBC excitability is also modulated in a stimulus-independent, top down manner. This mechanism may ultimately be regulated by the behavioral state of the animal and goes beyond the already known, stimulus dependent signaling through OCB collaterals.

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Figure captions

Figure 1 Carbachol application elicits transient depolarization and inward current in SBC. (A-C) Identification and characterization of SBC. **(A)** Maximum intensity z-projection of confocal stacks from a biocytin-streptavidin labeled SBC. Scalebar: 25 µm. **(B)** Current clamp recording of a P17 SBC; stimulus is shown below. SBCs typically fire 1-2 APs upon suprathreshold depolarizing currents and show a pronounced voltage sag upon hyperpolarizing currents (asterisk). Scalebars: 40 mV & 40 ms (top), 0.4 nA (bottom). **(C)** Synaptic currents recorded elicited by electrical stimulation of the auditory nerve in the same SBC as in (B), -60mV holding potential. The succession of two EPSCs show clear depression typical for SBC in vitro (stimulus artifacts removed). Scalebar: 500 pA & 5 ms. **(D-G)** Transient effects of carbachol-mediated nAChR activation. **(D)** Example traces of current clamp recordings with puff application of carbachol ("Carb", application time marked by black arrow). The transient depolarization elicited by the carbachol puff **(i)** was abolished when the slice had been superfused with D-TC, a general nAChR blocker **(ii)**, or with MLA, a specific α7 nAChR blocker **(iii)**. Puffing only the vehicle (ACSF) yielded no effect **(iv)**. Scalebars: 1 mV & 500 ms, apply to all traces. **(E)** Population data for SBC current clamp recordings. Asterisks indicate significant difference compared to the Carbachol condition ($p<0.01$, Kruskal-Wallis Test with Bonferroni post hoc test; Carbachol n=13, Carb+DTC n=8, Carb+MLA n=4, ACSF only n=5). **(F)** Example traces of voltage clamp recordings at -60 mV holding potential with carbachol application (application time marked by black arrow). SBC showed a transient inward current upon carbachol application **(i)** which was abolished under blocker wash-in of D-TC and MLA **(ii & iii, respectively).** No current was observed upon puff application of the vehicle only **(iv)** Scalebar: 50 pA for **(i)**, 20 pA for **(ii-iv)** & 500 ms (all). **(G)** Population data for SBC voltage clamp recordings. Asterisks indicate significant difference compared to Carbachol condition ($p<0.01$, Kruskal-Wallis test with Bonferroni post hoc test; Carbachol n=19, Carb+DTC n=7, Carb+MLA n=4, ACSF only n=4).

Figure 2 Cholinergic innervation of the Gerbil's CN. (Ai) Calretinin (CR) staining was used to distinguish CN subdivisions (A=AVCN, P=PVCN, NR=nerve root). SBC can be indirectly identified by visualization of their large somatic, CR-positive synaptic endbulb of Held terminal. **(Aii)** Staining with anti-choline acetyltransferase (ChAT) showed immunolabeling in all subdivisions of the CN, with increased signal intensity in the PVCN (arrowhead) und immunolabeling of cholinergic fibers arranged in stripes in the auditory nerve region (arrow). Scalebars: 250 µm. **(Bi)** Staining with antibodies against vesicular acetylcholine transporter (VAChT) showed strong immunolabeling in the AVCN and PVCN. This staining also revealed VAChT positive fibers with *en passant* swellings (white box in AVCN and arrows in PVCN). **(Bii)** Z-Projection of a high-magnification confocal stack recorded at the site indicated by the white square in (Bi)**,** double-staining with CR and VAChT. VAChT positive fibers (arrow) pass through the neuropil inbetween SBCs forming *en passant* swellings (arrowheads). Scalebars: 250 µm (i), 25 µm (ii). **(C)** Confocal image of a frontal section of the AVCN; double stained with CR and VAChT. In this cutting plane only few tangentially oriented labeled fibers were observed (arrow), instead single puncta-like signals were present. Scalebar: 25 µm. **(D)** VAChT staining in a sagittal VCN section with demarcation of the granular cell domain (region between dotted lines). Note the stronger VAChT signals in GCD (arrowheads) compared to the rest of the AVCN. VAChT signals can also be seen in the auditory nerve root (arrow). Scalebar: 100 µm. **(E)** Staining with Alexa-conjugated α-BTX yielded homogeneous distribution of BTX signals in the AVCN and the region of the auditory nerve root being left blank. **(F)** High magnification confocal image of CR and BTX double staining in the AVCN showed punctate signals in the neuropil surrounding the SBC. Scalebars: 250 µm (i) & 20 µm (ii). Orientation of slices: D = dorsal, R = rostral, M = medial, L = lateral (applies to all images).

Figure 3 Acetylcholine sets SBC's RMP through muscarinic receptors (A) Representative example trace of SBC depolarization during puff application protocol (see material and methods). The darker the trace, the later the recording took place. Small numbers and arrow right of the traces indicate

puff number. Note the persistent elevation of the RMP which tends to increase with repeated puff applications. **(B)** Course of RMP changes after 10 carbachol puff applications (0.1 Hz). Gray lines show the RMP courses of single cells (n=13). Circle markers show time-binned RMP averages of all cells: The horizontal error bars indicate the used time interval to calculate the mean, the vertical error bars denote standard deviation (middle of the crosshair). **(C)** Wash-in of 2 µM atropine (AT, red bar) strongly hyperpolarized the cells. This effect was reversible and RMP returned to baseline values after wash-out of AT (n=6, light red lines). Circle markers and errorbars show interval and standard deviation of time-binned RMP averages. **(D)** Wash-in of the specific mAChR blocker tolterodine (Tol; 100 nM, blue bar) showed a similar effect as AT wash-in (light blue lines ,n=3), crosshairs show intervals and standard deviation of time-binned RMP averages. Note that differences in the time-course of atropine vs. tolterodine effects are due to different perfusion systems used. **(E)** Carbachol application under atropine block. No depolarization occurred when mAChR were blocked. Light orange lines – single cells (n=5), circle markers: time-binned averages +/- STD of all cells. **(F)** Control experiment with application of the vehicle only (ACSF). No depolarization was visible after application. Light green lines - single cells (n=3), circle markers: time-binned average of all cells +/- STD. **(G)** Population data for all cells. Bars show mean of maximum RMP change. * – significant difference to Carbachol condition ($p<0.01$, Kruskal-Wallis test with Bonferroni post hoc test).

Figure 4 Pharmacological block of muscarinic AChR hyperpolarized the resting membrane potential of SBC at physiological temperature. (A). Resting membrane potential of n=6 SBC (thin gray lines) monitored for 20min at 37°C. Black open circle markes and black lines: mean ± SEM measured at 30s intervals. Horizontal black bar: interval of tolterodine wash-in. **(B)** Averaged RMP before (Control, "Ctrl", 0-4 min), during (tolterodine, "Tol", 4-14min) and after (Recovery, "Rec", 14-20min) wash-in of tolterodine. Gray markers connected by gray lines show individual SBC, black open circle markers show mean ± standard deviation of n=6 SBC. Black lines and asterisk show significant differences in

post-hoc testing, $p < 0.05$. **(C)** Membrane resistance R_m of n=6 SBC from the same measurements as in A, same presentation as in A. **(D)** Averaged R_m before, during and after wash-in of tolterodine, same presentation as in B.

Figure 5 Cholinergic modulation increases SBC spike probability in vitro without a loss in temporal precision. **(A)** Twenty-five repetitions of IVL stimuli were successively presented with 3 s interval. Gray triangles – input spikes **(B)** Example response of one SBC to the IVL stimulus paradigm. Red dots – failed AP, black dots – successful AP. **(C)** Excerpts from IVL Stimulus #18 under control conditions **(Ci)**, under cholinergic modulation via carbachol (Carb, **Cii**) and with elevated RMP through current injection (I_{inj}, **Ciii**). Note the increase in spike probability. Markers: Red dots – failed AP, black dots – successful AP. Scalebars: 20 mV & 50 ms (applies to Ci, Cii & Ciii). **(D)** Increase in spike probability under cholinergic modulation. **(Di)** Absolute change in spike probability for cholinergic modulation (Carb) and for RMP elevation through current injection (I_{inj}). Asterisks indicate significant difference ($p<0.01$, paired t-test). **(Dii)** Mean relative change of spike probability for all cells in (Di). Spike probability is increased twofold compared to control for both conditions. Asterisks indicate significant difference ($p<0.01$, paired t-test). **(E)** Changes in vector strength under cholinergic modulation and RMP elevation. **(Ei)** No significant change in absolute VS of SBC output for cholinergic modulation or RMP elevation was observed ($p>0.7$, paired t-test). Blue arrow denotes input-VS from the IVL stimuli. **(Eii)** Only a slight, but not significant ($p>0.7$, paired t-test) increase in mean VS could be seen for both conditions.

Figure 6 Carbachol increases SBC spike probability in vivo. (**A-F**) Example recordings of spontaneous spike rate in 6 different identified SBC units in vivo at 4 different carbachol concentrations (5-500 mM). Horizontal gray bars mark on- and offset of iontophoretic application. Spike rates are

normalized to the average of the first 5 seconds of spontaneous activity. Asterisks in A and B mark reduction of spontaneous spike rate by glycine control iontophoresis to 55% in A and 24% in B. (**G**) Grand average of spontaneous spike rate profiles over all units and carbachol concentrations (black line, n = 24), aligned on the onset of the iontophoretic current. Carbachol application resulted in an increase in spiking activity, while ACSF application had no effect (green line, n = 7). (**H**) Mean rate increase and temporal profile of spike rate is dependent on the concentration of the iontophoretic drug. Superimposed spike rate profiles for the different concentrations shown in the same colors as A-F, mean spike rate profile under ACSF iontophoresis shown in green. Note: for increased visibility only the segments during ionotophoretic application are shown here (0s = start of iontophoresis). (**I**) Comparison of spontaneous spike rates during iontophoretic drug application plotted as mean±standard deviation (colors same as A-H).

Figure 7 In-silico model of the functional relevance of cholinergic SBC modulation. (**Ai**) Example trace of simulated SBC membrane potential including sound driven excitation & inhibition and matched nicotinic input. Scalebars represent 100 ms and 10 mV. Red triangles mark “extra” action potentials that failed in the simulation of the same spike train without the nicotinic input. Red trace: shape of the nicotinic PSP, y-scale represents 2 mV for this trace. Gray bar: sound stimulus. Black arrowhead: onset of nicotinic event. (**Aii**) Peristimulus time histogram for 100 repetitions of Ai with (black line) and without (green line) nicotinic input. Gray bar depicts stimulus duration and red line depicts nicotinic PSP as in A. Simulation conditions were 3 kHz, 20 dB SPL, CF=3 kHz. (**Aiii**) Rate-level function for -10 to 75 dB SPL, 3 kHz. CF=3 kHz. Dark-gray line shows identical spontaneous rate for all condition. Colors as in Aii. Light-gray curve shows input RLF. Inset shows first four levels (-10, -5, 0, 5 dB SPL; scale of inset: x-axis -16 to 6 dB SPL, y-axis 50 to 70 spikes/s) (**Aiv**) Period histograms of spiketimes for the nicotinic condition, colors as in Aii, normalized to the maximal amplitude of the control condition. Arrows show preferred phase. Simulation conditions shown here were 1682 Hz,

60 dB SPL, CF=1200 Hz (see gray vertical line in Av). (**Av**) Relative change in vector strength to 60 dB SPL tones of different frequencies for nicotinic condition compared to control condition. Colors as in Aii. Filled circles depict significant phase-locking. Gray vertical line shows the frequency of the period histogram in Aiv. (**Bi**) Example trace of the same spike train simulated at the three modulatory conditions. Red: simulated atropine, black: simulated carbachol, green: control, Circles mark occurrence of action potentials under the three conditions. Scalebars represent 25 ms and 10 mV. (**Bii**) Rate-level functions for the three modulatory conditions in Bi, colors as in Bi. Light-gray curve shows input RLF. Simulation conditions were 1.2 kHz, CF=1.2 kHz. Arrows show spontaneous rate per condition. (**Biii**) Frequency response area for 9 stimulus levels and 21 frequencies, colors as in B. Dashed contour for each color shows +5 Hz increase in response spike rate vs. spontaneous rate, inner, solid contour shows +50 Hz increase in response spike rate vs. spontaneous rate. CF=1.2 kHz. (**Biv**) Period histograms of spiketimes for the three modulatory conditions, colors as in Bi, normalized to the maximal amplitude of the control condition. Arrows show preferred phase. Simulation conditions were 1682 Hz, 60 dB SPL, CF=1200 Hz. (**Bv**) Relative change in vector strength to 60 dB SPL tones of different frequencies for three modulatory conditions, compared to control condition. Colors as in B. Filled circles depict significant phase-locking. Gray vertical line shows the frequency of the period histogram in Biv.