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Muscarinic Acetylcholine Receptor Subtype Expression in Avian Vestibular Hair Cells, Nerve Terminals and Ganglion Cells

Gang Q. Li^{1,2}, Golda A. Kevetter^{1,2}, Robert B. Leonard^{1,2}, Deborah J Prusak³, Thomas G. Wood^{3,4}, and Manning J. Correia^{1,2}

1 Department of Otolaryngology, University of Texas Medical Branch at Galveston, Galveston Texas, 77550-1063 U.S.A.

2 Department of Neuroscience and Cell Biology, University of Texas Medical Branch at Galveston, Galveston Texas, 77550-1063 U.S.A.

3 Sealy Center for Molecular Medicine, University of Texas Medical Branch at Galveston, Galveston Texas, 77550-1063 U.S.A.

4 Department of Molecular Biology and Biochemistry, University of Texas Medical Branch at Galveston, Galveston Texas, 77550-1063 U.S.A.

Abstract

Muscarinic acetylcholine receptors (mAChRs) are widely expressed in the central and peripheral nervous systems and play an important role in modulating the cell activity and function. We have shown that the cholinergic agonist carbachol reduces the pKir2.1 ionic currents in native vestibular hair cells. We have cloned and sequenced pigeon mAChR subtypes M2–M5 and we have studied the expression of all five mAChR subtypes (M1–M5) in the pigeon vestibular end organs (semicircular canal ampullary cristae and utricular maculae), vestibular nerve fibers and the vestibular (Scarpa's) ganglion using tissue immunohistochemistry (IH), dissociated single cell immunocytochemistry (IC) and Western blotting (WB). We found that vestibular hair cells, nerve fibers, and ganglion cells each expressed all five (M1–M5) mAChR subtypes. Two of the three odd-numbered mAChRs (M1, M5) were present on the hair cell cilia, supporting cells and nerve terminals. And all three odd numbered mAChRs (M1, M3 and M5) were expressed on cuticular plates, myelin sheaths, and Schwann cells. Even-numbered mAChRs were seen on the nerve terminals. M2 was also shown on the cuticular plates and supporting cells. Vestibular efferent fibers and terminals were not identified in our studies. Results from WB of the dissociated vestibular epithelia, nerve fibers and vestibular ganglia were consistent with the results from IH and IC. Our findings suggest that there is considerable co-expression of the subtypes on the neural elements of the labyrinth. Further electrophysiological and pharmacological studies should delineate the mechanisms of action of muscarinic acetylcholine receptors on structures in the labyrinth.

Keywords

avian; vestibular peripheral system; G-protein; immunohistochemistry; immunocytochemistry; Western blotting

Correspondence to: Manning J. Correia Ph.D., Room 7.102, Blocker Medical Research Building, UTMB, Galveston, Texas 77555-1063, E-mail: mjcorrei@utmb.edu, Phone: 409-772-2708, Fax: 409-772-2694

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Two decades ago, the first muscarinic acetylcholine receptor subtype (mAChR) was successfully cloned (Kubo et al., 1986a). The cDNAs from the four other known subtypes were subsequently cloned (Kubo et al., 1986b; Peralta et al., 1987a; Peralta et al., 1987b; Bonner et al., 1987; Bonner et al., 1988). Muscarinic receptors belong to the G-protein coupled receptor family. The receptors have seven transmembrane domains. The residues in the third intracellular loop of the protein contribute to the structural diversity between the 5 different mAChR subtypes (Wess et al., 1997; Wess, 1996; Hulme et al., 1990). Based on their sensitivity to pertussis toxin, similar biochemical characteristics and their intracellular cascades (Caulfield, 1993; Eglén and Nahorski, 2000), the five subtypes can be subdivided into two groups. The first group is composed of M1, M3 and M5. These subtypes are sensitive to pertussis toxin and they are coupled to G α q/11 to activate phospholipase C and downstream intracellular cascades. The second class consists of M2 and M4. These subtypes are not pertussis toxin sensitive and they are preferentially coupled to G α i/o proteins to inhibit adenylate cyclase and influence subsequent downstream pathways.

All five muscarinic receptor isoforms are widely expressed throughout the central and peripheral nervous systems. Transgenic mice lacking genes that encode for each of the mAChR subtypes have provided some useful generalizations about their function (Wess et al., 2003b). The M1 receptor appears to be associated with locomotor function and higher cognitive processes such as learning and memory (Miyakawa et al., 2001; Wess, 1996). M2 receptors contribute to skeletal and smooth muscle contraction, analgesia (Wess et al., 2003a) and thermal regulation (Stengel et al., 2000). M3 receptors are involved in smooth muscle contraction, glandular secretion, food intake and weight control (Yamada et al., 2001b; Matsui et al., 2000; Caulfield, 1993). M4 receptors contribute to locomotor activity and ACh release in the striatum (Zhang et al., 2002; Gomeza et al., 1999). M5 receptors modulate dopamine release in the central nervous system and they are involved in vasculature vasodilatation (Yamada et al., 2001a; Vilario et al., 1990; Weiner et al., 1990; Basile et al., 2002; Yamada et al., 2003; Fink-Jensen et al., 2003; Wess et al., 2003b).

In birds, preliminary pharmacological studies indicate that mAChRs located in the central nervous system are associated with vision, homing, navigation, metabolism and central thermoregulation (de Azevedo et al., 2002; Kohler et al., 1996; Chawla et al., 1975). However, the role of each of the mAChR subtypes has not been clearly established in either the central or peripheral nervous systems (Kohler et al., 1995; Dietl et al., 1988).

Muscarinic receptor subtypes have been shown to be expressed in cochlear neurons, supporting cells (Wangemann et al., 2001; Drescher et al., 1992; Khan et al., 2002) and central auditory neurons (Chen et al., 1995). While message for mAChR subtypes has been demonstrated in peripheral vestibular neurons (Wackym et al., 1996; Wackym et al., 1991; Anderson et al., 1997; Wackym et al., 1995; Ishiyama et al., 1997), protein expression has not been shown previously. Acetylcholine, however, is the major neurotransmitter released from vestibular efferent terminals, the endings of fibers coursing centrifugally from the brain to synapse on sensory hair cells and afferent nerve terminals (Iurato et al., 1971; Gacek et al., 1965; Goldberg and Fernandez, 1980).

Pioneering physiological studies using the application of cholinergic agonists in the isolated labyrinth or the isolated semicircular canal frog preparations produced both inhibitory and facilitatory effects on primary afferent activity (Bernard et al., 1985; Guth et al., 1986; Norris et al., 1988). Subsequent patch clamp studies of dissociated individual frog vestibular hair cells indicated that “atropine-preferring” (Norris et al., 1988) or “muscarinic-like” (Guth and Norris, 1996) receptors were expressed on the vestibular hair cell membrane (Housley et al., 1990; Yoshida et al., 1994).

We demonstrate here that the application of carbachol reduces pKir2.1 currents in pigeon vestibular hair cells. In a previous study (Correia et al., 2004), it was shown that inhibition of pKir2.1 currents depolarized the hair cell membrane potential. Furthermore, we demonstrate for the first time, the expression and co-expression of M1–M5 in supporting and neural cells of the pigeon vestibular periphery. Understanding the functional role of each receptor subtype in isolation or in combination will be the challenge of future experimentation.

Partial preliminary results were published as an abstract (Li et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

Adult (6–40 wk old) white king pigeons (*Columba livia*) of either sex were used for the present studies, including electrophysiology, vestibular peripheral anatomy and PT-PCR analysis. The antibody studies of each of the muscarinic receptor subtypes (M1–M5) were performed using frozen section immunohistochemistry (IH), dissociated single cell immunocytochemistry (IC) and Western blotting (WB). The number of animals/subtype used: in the frozen section studies ranged from 4–7; in the isolated hair cell studies ranged from 3–7 and in the Western blotting studies ranged from 4–10. In no case did fewer than 3 animals provide data for each subtype and each method. The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee.

Electrophysiology

Pigeons were anesthetized using Nembutal, 40 mg/kg iv, and ketamine, 60 mg/kg im (supplementary doses as needed). While deeply anesthetized, the vestibular end organs were removed as described previously (Correia et al., 1989), placed into a D-MEM solution (pH 7.4, osmolarity = 320 mosmol/kg H₂O) supplemented with (in mM): 24 NaHCO₃, 15 PIPES and 1.5% fetal calf serum and kept in an incubator at 37°C. The atmosphere inside the incubator was saturated with a 95% CO₂/5% O₂ mixture (Ricci et al., 1996b). The incubation environment prolonged hair cell life to 4–6 hrs. After 1–2 hrs in the incubator, the vestibular end organs were dissected, trimmed well, oriented and embedded within 4% agar. The agar block containing the ampulla was placed in a partially frozen high Mg²⁺ solution consisting of (in mM): 3 KCl, 145 NaCl, 0.1 CaCl₂, 7.5 MgCl₂, 15 HEPES, 10 glucose, 0.283 sodium ascorbate and 2 Na⁺ pyruvate and sliced using a Vibratome® 1000 sectioning system (Ted Pella Inc., Redding, CA, USA). The plane of the section was parallel to the long axes of the crista and the thickness of each slice was 150–250 μm.

The individual sliced section was put in a recording chamber bathed with an oxygen saturated external solution comprised of (in mM): 3 KCl, 145 NaCl, 2 CaCl₂, 1 MgSO₄, 15 HEPES, 10 glucose, 0.283 sodium ascorbate and 2 Na⁺ pyruvate and stabilized by a weighted nylon mesh net for electrophysiological recording (Masetto and Correia, 1997a; Weng and Correia, 1999). The slices were viewed using a Zeiss Axioskop compound upright microscope and DIC optics including an Optovar magnifier and a 40X water-immersion objective. The temperature of the bath was 23°C and continuously changed at a rate of ~750 μl/min.

The patch pipettes contained (in mM): 140 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES and 11 EGTA. The pipette solution was titrated to a pH of 7.4 with KOH/HCl and to an osmolarity of 320 mosmol/kg H₂O.

The borosilicate pipettes (0.75 ID, 1.50 OD, no. 1B150F-3; World Precision Instruments, Sarasota, FL, USA) were pulled on a P-2000 puller (Sutter Instruments, Novato, CA, USA), then firepolished on a MF-83 microforge (Narishige USA Inc., Greenvale, NY, USA). Electrode capacitance was greatly reduced by the following procedure which obviated further

pipette coating. The glass blanks containing a thin filament were initially washed with Chromerge (VWR International, Cat. # 21865-000, West Chester, PA, USA), silanized with 5% dimethyldichlorosilane (Sigma, Cat. # 40140, St. Louis, MO, USA) in chloroform (Fisher, Cat. # C298-500, Pittsburgh, PA, USA), then subsequently washed multiple times with ddH₂O and dry heat sterilized at 240°C. The whole cell recording electrode impedance was typically 2–5 MΩ.

Whole cell currents were amplified 1X using an Axopatch 200 amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). The currents were filtered at 2 kHz using the amplifier's Bessel filter then sampled at 5 kHz. Series resistance and capacitance were compensated between 80 and 98% with a typical value of 95%. No online leak subtraction was performed. Subsequently, in some cases, leak subtraction was performed digitally. Current and voltage were measured and controlled through a DigiData 1200 Interface (AD/DA converter; Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). Resting membrane potential was measured as the zero-current voltage (V_z) in current-clamp mode. Cell input resistance (R_m) was calculated by measuring the average current evoked by a series of seven depolarizing pulses (10 mV in amplitude and 250 ms in duration) from a holding potential of –60 mV (Fig. 1). This voltage series yielded the largest R_m . Because no time-dependent currents were evident, we considered the current measured in response to the above protocol to be leakage current. This leakage current was subsequently subtracted from the current traces. Voltages were corrected for the liquid junction potential (~3 mV negative inside the pipette). Although current-voltage (I – V) plots were not corrected for the voltage drop across the residual series resistance, all values of peak (G_p) and steady-state (G_s) slope conductance were corrected for the residual series resistance.

The cholinergic agonist carbachol was superfused onto the cell using a pressurized computerized superfusion system (model DAD-12; ALA Scientific Instruments, Westbury, NY, USA). The superfusion pipette (100 μm bore) was placed ~30 μm from the cell; and the flow rate was ~20 μl/min.

The patch clamp data were gathered using Clampex V8 (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA), and Fig. 1 was produced using Clampfit V8 (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) and OriginPro v7.5 (OriginLab, Northampton, MA, USA)

Transmission electron microscopy

Pigeons were anesthetized and perfused by *in vivo* transcardiac bilateral carotid catheterization (Eden and Correia, 1981) and pump driven perfusion. The perfusate was 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4). Following overnight submersion of the head in the same concentration of glutaraldehyde, the anterior ampullae were dissected free of the head and post fixed for 1 hr in 1% osmium tetroxide (dissolved in ddH₂O) (Correia et al., 1985). After 2X H₂O rinses, the ampullae were stained with 1% uranyl acetate for 2 hrs and then dehydrated through a graded series of ethanols (70% - 3X for 15 min each time (ET), 95% - 1X for 30 min and 100% - 2X for 1hr ET). Then the tissue was immersed in propylene oxide (2X for 30 min ET) alone and then overnight in a 1:1 mixture with Epon. Subsequently, the tissue was blocked and oriented so that thin sections could be made through the long axis of the crista. The thin sections were viewed and photographed using a Philips 200 or a Zeiss 10 TEM (Fig. 2).

RT-PCR cloning of M2-M5 open reading frames (ORFs)

Total RNA from pigeon brain was isolated using guanidinium thiocyanate (Chomczynski and Sacchi, 1987). Synthesis of cDNA and PCR amplification of ORFs for M2-M4 were performed as described previously (Correia et al., 2004) using primers listed below. M1 ORF amplification

and cloning was unsuccessful. The M5 ORF was initially amplified as separate 5' (824bp) and 3' (932bp) DNA fragments using conditions identical to those used for M2-M4 and the M5 primers (see below). The full length ORF was generated by annealing the purified DNA fragment followed by primer extension and then amplification using the 5' forward and 3' reverse M5 primers.

M2 Forward – 5'GAATTCATGAATAACTCAACGTACATAAACTCTTC 3'

M2 Reverse – 5'GTCGACTTACCTTGTGTGCTCCTATATTCTTGTAAAT 3'

M3 Forward – 5' AAGCTTCGATGCTGACACACTACCAGTTGTGTTTC 3'

M3 Reverse – 5' GTCGACTATGAAGCCTCCCTAGGGATCCG 3'

M4 Forward – 5'ATGCACAACCTCTCCGCGCAGCC 3'

M4 Reverse – 5' TTATCTGGCCGTGCCAATGTTTCCTG 3'

M5 5' Forward – 5' ATGGAAGTAAATCTATTCAGCAATTCTAC 3'

M5 5' Reverse – 5' CGACTGGATGAAGACCAGGAAGCC

M5 3' Forward – 5' GAGACTGAGAAACGTACCAAGGACC 3'

M5 3' Reverse – 5' TTATGGCATTGAGTGTGTCCTGC 3'

Primer design was based upon the nucleotide sequence of the respective chicken ORFs for M2–M5 (M73217, L10617, J05218 and AF201960, respectively). The PCR products for M2–M4 were cloned into a PCR2.1 vector. The initial PCR products for M5 were cloned into a pUC vector digested with *Sma*I and treated with calf intestine phosphatase (CIP). The final full length M5 ORF was cloned into PCR2.1. The M2–M5 clones were confirmed by DNA sequence analysis. The GenBank accession numbers for the nucleotide sequences of pigeon M2–M5 are AY838767, AY838768, AY838766 and DQ357059, respectively.

Pigeon M2–M5 sequence analysis

The epitope sequences of the commercial mammalian anti- M2, M3, M4 and M5 antibodies were obtained from the manufacturers and aligned (using clustal W analysis) for comparison to pigeon muscarinic receptor subtype sequences. The alignment was done using the Megalign module of DNASTar (DNASTar, Madison, WI, USA). The results are shown in Table 1. Pigeon and human M2–M5 sequences were clustered using clustal W and phylogenetic analyses (Accelrys Gene 2.0, Accelrys, San Diego, CA, USA). The probability of serines, threonines, tyrosines and asparagines being phosphorylation and glycosylation sites were obtained for the sequence DQ357059 (Pigeon M5) using the website www.cbs.dtu.dk/databases. Only those residues having a probability of 0.5 or greater were recorded and indicated in Fig. 3.

Frozen section tissue preparation

Pigeons were anesthetized as described above. While deeply anesthetized, each pigeon's chest was opened to expose the heart. Two cannulae (14 Gauge, Harvard Apparatus, Holliston, MA, USA) were inserted into the left and right carotid arteries through the left ventricle (Eden and Correia, 1981). The right atrium was opened. About 200–300 ml of warm (36°C) 0.9% NaCl solution was perfused through the upper body. To improve the perfusion efficiency, about 10 mg NaNO₂ was added in the saline solution to dilate the arteries. Then, 500–600 ml cold (4°C) 4% paraformaldehyde solution (freshly made) was perfused (gravitationally driven) for one hour. The head of each pigeon was removed and submerged in fixative and stored at 4°C overnight after exposing a small part of the membranous semicircular canals of the vestibular end organs and the skull covering the brain. The next day, the membranous semicircular canals and utricles were excised and dissected apart. All ampullae and utricles were put into PBS w/

20% sucrose until they sank. Then they were immersed in 3% gelatin w/ 20% sucrose for 15 seconds. Subsequently, the sensory receptors were embedded in 10% gelatin w/ 20% sucrose; chilled in the refrigerator for one hour; blocked, trimmed and fixed in 4% paraformaldehyde w/ 20% sucrose overnight in the refrigerator. The vestibular ganglia were also taken out and treated in the same way.

A sliding microtome (Model 860, American Optical Corp., Buffalo, NY, USA) was used to cut sections 20 μm thick. Each of the well-fixed gelatin embedded ampullae, utriculi, Scarpa's ganglia and hippocampus tissue were oriented on the microtome plate and frozen using ground dry ice. The plane of the sections was parallel to the long axis of the crista, perpendicular to the long axis of the utricle and parallel to a horizontal section through the vestibular ganglion.

As positive controls, two gerbils and one rat were perfused with the same fixatives. Brains were dissected and 40 μm sections through the hippocampus and striatum were cut and reacted concurrently with pigeon tissues.

Isolated single cell preparation

As described previously (Correia et al., 1989), the membranous vestibular end organs were removed from anesthetized pigeons then immediately put into D-MEM/PIPES solution (pH 7.4, Osm=320 mosmol/kg H_2O) supplemented with 24 mM NaHCO_3 , 10 mM PIPES and 1.5% fetal calf serum, which was kept in an incubator whose atmosphere was maintained at 37°C and saturated with a CO_2/O_2 mixture. The incubation environment prolonged hair cell life for 4–6 hrs but killed supporting cells thereby facilitating dissociation (Ricci et al., 1996a). The end organs were dissected in 4°C low Ca^{2+} saline (containing in mM: 0.1 CaCl_2 , 110 NaCl , 2 KCl , 2 MgCl_2 , 3 D-Glucose and 10 HEPES at pH 7.25) to separate the cristae and utriculi (Hirono et al., 2004). The cristae and utriculi were put in low Ca^{2+} saline w/ 1 mM EGTA (Sigma, Cat. # E3889-25G, St. Louis, MO, USA) for 15 min. During the EGTA/low Ca^{2+} incubation, the membrane overlying each crista and macula was opened to increase the efficiency of subsequent enzyme digestion. The end organs were then placed in protease XXIV (65 $\mu\text{g}/\text{ml}$ in low Ca^{2+} saline, Sigma, Cat. # P8038-250MG, St. Louis, MO, USA) for 20 min. Protease XXIV facilitated easy removal of the otolithic membrane and cupula and therefore decreased the damage to the hair cell cilia (Ricci et al., 1996a). Parenthetically, it has been reported that protease XXIV used for hair cell isolation eliminates the $\alpha 9$ -nicotinic acetylcholine receptor (nAChR) mediated response (Holt et al., 2001). However, it is not clear whether this effect is on the receptor (nAChR) or the effector (SK ion channel). No proteolytic effects on mAChR subtypes have been reported to our knowledge. Next, the end organs were put in deoxyribonuclease I (100 $\mu\text{g}/\text{ml}$ in low Ca^{2+} saline, Worthington, Cat. # X5D8021, Lakewood, NJ, USA) for 5 min, followed by bovine serum albumin (1 mg/ml in low Ca^{2+} saline, Fisher, Cat. # BP1605-100, Pittsburgh, PA, USA) for 5 min to severely attenuate the enzyme reaction. In a glass chamber containing 60 μl low Ca^{2+} saline, a thin glass wisp was used to dislodge hair cells from the epithelia of the cristae and utriculi through a gentle mechanical agitation. Following subsequent trituration, the hair cells were plated in a channel of μ -Slide (Ibidi GmbH Integrated BioDiagnostics, Cat. # 80601, München, Germany). The bottom of the channel (a slide) was coated with concanavalin A (5 mg/ml, Sigma, Cat. # C2010-250MG, St. Louis, MO, USA). To fix the isolated hair cells, 2.25% formaldehyde (Electron Microscopy Sciences, Cat. #15682, 37%, Hatfield, PA, USA) and 0.75% glutaraldehyde (Electron Microscopy Sciences, Cat. #16310, 50%, Hatfield, PA, USA) in PBS (containing in mM: 137 NaCl , 2.7 KCl , 4.3 Na_2HPO_4 and 1.4 KH_2PO_4 at pH 7.4) was gently added and kept at room temperature (RT) for 30 min. Ganglion cells were isolated and treated in the same way.

Immunohistochemistry and immunocytochemistry

Frozen sections were rinsed in phosphate-buffered saline (0.1 M pH 7.4 PBS; 0.9% NaCl) for 10 min before they were incubated for 1 hr in 3% normal goat serum diluted in the working buffer (PBS w/ 0.3% triton X-100 (Sigma, Cat. # T6878 [X100], St. Louis, MO, USA). The sections were kept at 4°C for 36–48 hrs in primary antibody diluted in working buffer. Subsequently, the sections were incubated in one of the fluorescent tagged secondary antibodies diluted in working buffer for 2 hrs at RT. For double labeling, the sections were incubated with two different primary antibodies and appropriate secondary antibodies. Between each step, the sections were rinsed 3X for 10 min ET in PBS. Once the reaction was finished, the sections were rinsed 2X for 10 min ET in PBS and 1X for 10 min in distilled water. Then, they were mounted on gelatin-coated glass slides. After the sections dried at RT overnight, they were cover-slipped.

For the diaminobenzidine (DAB) reaction, a biotinylated secondary antibody and VECTASTAIN® ABC reagents (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) were used according to the manufacturer's protocol. Sections were rinsed 2X for 10 min ET with PBS and 1X for 10 min ET with acetate buffer. The immunoreactivity of mAChR subtypes was visualized using 0.05% DAB (3,3'-Diaminobenzidine tetra-hydrochloride, Sigma, Cat. # D5637-1G, St. Louis, MO, USA) dissolved in 0.1 M acetate buffer containing 1.75% nickel ammonium sulfate (Fisher, Cat. # N-48, Pittsburgh, PA, USA) and 0.0003% H₂O₂. Sections were mounted on gelatin-coated microscope slides, dried overnight and cover-slipped with Permount* mounting medium (Fisher, Cat. # SP15-500, Pittsburgh, PA, USA).

In some cases adjacent frozen sections were stained with 1% osmium tetroxide (OsO₄) for one hour to stain myelin.

For IC using isolated vestibular hair cells and ganglion cells, samples were rinsed with PBS 3X for 10 min ET after fixation and blocked 1 hr at RT in the blocking solution (PBS, 3% goat serum and 10 mg/ml bovine serum albumin). The isolated hair cells and ganglion cells were then incubated 48 hrs at 4°C with primary antibodies diluted in the blocking solution. After rinsing 3X for 10 min ET in PBS, tissues were incubated with secondary antibodies in the blocking solution for 2 hrs at RT. Following 2X PBS for 10 min ET and 1X for 10 min distilled water rinses, mounting medium was applied to offer enhanced resistance to photobleaching and the slides were cover slipped. Two types of mounting medium were used for fluorescent microscopy: one was VECTASHIELD® mounting medium (Vector Laboratories, Inc., Cat. # H-1400, Burlingame, CA, USA), which was used mainly for the isolated cells and the second, Prolong® Gold antifade reagent (Invitrogen, Cat # P36930, Carlsbad, CA, USA), was used mainly for the frozen sections.

The concentrations of the primary antibodies were 1:500 for anti-M1, 1:200 for anti-M2, 1:200 for anti-M3, 1:200 for anti-M4 and 1:400 for anti-M5. The concentrations of the secondary antibodies, which were conjugated with fluorescent probes (Alexa™ probes; see above), were 1:500 for either Goat anti-Rabbit IgG or Goat anti-Mouse IgG.

Samples were viewed using either a confocal microscope (LSM 510 Meta, Zeiss, Thornwood, NY, USA) or a light microscope (Olympus BX51, Olympus, Tokyo, Japan). The Zeiss LSM Image Browser program (v 3.5.0.376) was used to process the optical sections of the frozen tissue slices and dissociated cells. The tissue and cells were excited with 488 nm and 543 nm wavelengths. Stacks and single optical sections (0.5 and 0.9 µm thick) of the cells and tissue were stored on disk. Light microscopic images of DAB and OsO₄ stained sections were captured using an Olympus DP 70 camera and DP Controller 2.1.1.183 software (Olympus, Tokyo, Japan). Figures containing or based on photomicrographs were made using Photoshop CS2 (v 9.0.1) and Illustrator CS2 (v 12.0.1) (Adobe Systems Inc, San Jose, CA, USA). No

manipulations except minor adjustments to brightness and contrast were made to any of the captured images.

Western blotting

Four types of cell lysates were obtained. The first consisted of dissociated cells and tissues from the vestibular end organ's neuroepithelia (NE). This lysate contained mostly hair cells but possibly some supporting cells and nerve terminals (calyces and boutons). The second lysate was from nerve fibers attached to the neuroepithelia of the end organs and nerve bundles peripherally connected to Scarpa's ganglia (NF). The third lysate consisted of dissociated ganglion cells from dissected vestibular ganglia (VG). The fourth lysate was from the hippocampus region of the pigeon brain (used as controls, H). After sample collection was done, each lysate was centrifuged at 2000g at 4°C for 5 min and the supernatant was removed. The RIPA buffer (see Millipore/Upstate protocol: RIPA Buffer: Preparation of Modified Radioimmunoprecipitation (RIPA) Buffer) was added and mixed with samples by vortexing the solution gently every 10 min for 30 min while keeping the samples on ice. Then, 8000g centrifugation was applied at 4°C for 20 min. The supernatants were transferred to clean tubes and total protein concentration was measured using Micro BCA Protein Assay Kits (Pierce Biotechnology, Inc., Cat # 23235, Rockford, IL, USA) and an ELx800 Microplate Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA). The average concentration (mean $\pm$ SD) for the total protein was: Neuroepithelium -0.59 ± 0.1 μ g/ μ l (60 μ l, n=6); nerve fibers -0.55 ± 0.16 μ g/ μ l (60 μ l, n=6); ganglion -0.56 ± 0.1 μ g/ μ l (75 μ l, n=6) and hippocampus -3.4 ± 0.38 μ g/ μ l (500 μ l, n=4). An aliquot of (8 μ g) from each cell lysate was mixed with 6X sample buffer, boiled in a water bath for 10 min, then electrophoresed through a 10% Tris-HCl gel (at 40 mA for 80 min) (Bio-Rad, Cat # 345-0009, Hercules, CA, USA). The protein product was transferred electrophoretically (at 100 V for 90 min) on to nitrocellulose membranes (Bio-Rad, Cat # 162-0145, Hercules, CA, USA). The membranes were blocked with Tris-Buffered Saline Tween-20 (TBST, 25 mM Tris, 0.15 M NaCl and 0.05% Tween-20 at pH 7.2) containing 5% non-fat milk for 1 hr at RT. Blots were either incubated with the muscarinic receptor antibodies or the corresponding pre-adsorption control solutions overnight at 4°C. After washing 3X for 10 min ET with TBST, the blots were reacted with the appropriate horseradish peroxidase (HRP) conjugated IgG for 1 hr at RT. After rinsing the blots 3X for 10 min ET with TBST, they were incubated using SuperSignal® West Femto Maximum Sensitivity Substrate working solution (Pierce Biotechnology, Inc., Cat # 34095, Rockford, IL, USA) for 5 min. Then, the excess solution was drained and the blots were covered with clear plastic wrap and were exposed to Hyperfilm™ ECL film (Amersham Biosciences UK Ltd, Cat # RPN1674K, Buckinghamshire, UK) for 15–60 seconds depending on the band intensities. The films were developed in an automatic developer and the resulting negatives were scanned using an EPSON Perfection model 4990 scanner. The resulting digital files were saved for further analysis. As with the IC and IH, primary antibody negative controls, secondary antibody negative controls and pre-absorption controls were performed.

All antibodies were diluted in 1% non-fat milk-TBST solution. The concentration of the anti-M1 was 1:1,000; the anti-M2 was 1:500; the anti-M3 was 1:500; the anti-M4 was 1:2,000; and the anti-M5 was 1:1,000. The goat anti-rabbit conjugated HRP IgG was diluted into 1:10,000. Goat anti-mouse conjugated HRP IgG was diluted into 1:20,000.

Antibodies

The primary antibodies included rabbit anti-M1-mAChR polyclonal antibody (Biodesign International, Cat. # Q4A173R, Saco, ME, USA), rabbit anti-M2-mAChR antibody (Alomone Labs Ltd., Cat. # AMR-002, Jerusalem, Israel), rabbit anti-M3-mAChR antibody (Alomone Labs Ltd., Cat. # AMR-006, Jerusalem, Israel), mouse anti-M4-mAChR monoclonal antibody (Chemicon International, Cat. # MAB1578, Temecular, CA) and rabbit anti-M5-mAChR

antibody (Bioscience International, Cat. # Q4A871R, Saco, ME, USA). Control antigens for M2 and M3 antibodies from Alomone Labs were included with the purchase of the antibodies. Blocking peptides for M1 (Cat. # A4A730H) and M5 (Cat. # A4A738H) were purchased from Bioscience International. M4 purified protein, used as a blocking peptide (Cat. # sc-4505 WB), was purchased from Santa Cruz Biotechnology, Inc. Molecular and pharmacological properties of the antibodies are detailed in Table 1.

The secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA). They included Alexa^{TM} 488 and Alexa^{TM} 594 labeled Goat anti-Mouse IgG and Alexa Fluor® 488 and Alexa Fluor® 594 labeled Goat anti-Rabbit IgG. In Western blotting experiments, ImmunoPure® Peroxidase conjugated Goat Anti-Rabbit HRP and Goat Anti-Mouse HRP (Pierce Biotechnology, Inc. Rockford, IL, USA) were used.

Negative controls

Several kinds of controls were carried out for IH and IC immuno-reactions and Western blotting. First, absence of antibody was used to control for tissue or cell autofluorescence (for IH and IC reactions). Second, primary antibodies were replaced by normal serum, followed by the completion of the protocol. This controlled for nonspecific fluorescence. Third, secondary antibodies were deleted and the reaction completed. Fourth and most robust, pre-absorption controls were performed for IH and WB, antigens (M1, M2, M3 and M5) or pure protein (M4) were pre-incubated with the corresponding antibodies with the ratio of 6:1 (M2 and M3), 3:1 (M1 and M5) and 1:10 (M4) by weight and kept at 4°C for at least 24 hrs.

Anatomical nomenclature

Based on anatomical features, types of hair cells present and the cadre of ionic currents in the hair cells, the pigeon crista and macula can be divided into zones (Kevetter et al., 2000; Masetto and Correia, 1997a; Masetto and Correia, 1997b; Weng and Correia, 1999). In the crista, when sectioned along its long axis, three zones naturally appear in each hemi-crista on either side of the eminentia cruciata. Zone 1 (I), containing type II hair cells, extends from the planum semilunatum to the region where there are almost exclusively type I hair cells (zone 2 (II)). Zone 3 (III) extends from the central apical region of the crista about 150 μ m and contains type II and type I hair cells. Zone 2 (II) is between zones 1 and 3 and contains mostly type I hair cells found singly or in multiples in calyces. Zones are also present in the pigeon utricular macula. In the striolar region, a zone (S) of cells is present that is comprised of a band of type I hair cells bordered by a several rows of type II hair cells only (Jorgensen and Anderson, 1973). In the remaining surface of the macula, the extrastriolar zone (ES), there are only type II hair cells. The zones from which data were obtained are identified in subsequent figures.

RESULTS

Patch clamp recordings

The application of carbachol, a cholinergic agonist, modulated (reduced) the ionic currents through an inward rectifier channel, pKir2.1, found in pigeon vestibular hair cells (Correia et al., 2004). Fig. 1 shows typical traces from a type II hair cell in a vestibular epithelial slice. Fig. 1Aa shows control traces. Fig. 1Ab presents traces acquired following application of 2 μ M carbachol for 30 seconds. A reduction in the amplitude of the inward currents by ~30% (Kir2.1) can be seen. The outward current traces do not show reduction. Following a 9 min wash with normal saline bath solution the traces recovered (Fig. 1Ac) to pre carbachol levels. The current (I) – voltage (V) plot illustrated in Fig. 1B documents the effects of the cholinergic agonist. The outward current at voltages > -50 mV are due to activation of other channels found in pigeon type II vestibular hair cells (Lang and Correia, 1989). Next we determined the source of the acetylcholine signaling molecule in the vestibular neuroepithelium.

Electron microscopy

Three hundred thirty eight photomicrographs obtained from thin sections through zones 2 and 3 of the epithelium of the pigeon's anterior semicircular canal crista were studied. The sections were cut in a plane parallel to the long axis of the epithelium. Previously, the photomicrographs had been used to characterize hair cell-afferent terminal synapses (Correia et al., 1985). In this study, the photomicrographs were examined for synaptic complexes containing: a vesiculated pre-synaptic bouton (efferent terminal), pre-synaptic electron density (or a synaptic cleft) and a post-synaptic cistern. These structures are the signature of vestibular efferent synapses (Wersall, 1956). Forty four synaptic complexes were found that met all of these criteria. Type I hair cells and their calyces, type II hair cells and primary afferent bouton terminals were identified. The following distribution was obtained for the 44 synapses. 61% (27/44) involved synapses between efferent terminals and type II hair cells; 2% (1/44) involved synapses between efferent terminals and type I hair cells; 25% (11/44) involved synapses between efferent terminals and type I hair cell calyces; and 11% (5/44) involved synapses between efferent terminals and primary afferent dendrites and terminals. Fig. 2 illustrates each of these synaptic complexes.

Molecular structure of muscarinic receptor isoforms

Having determined the distribution of efferent projections onto hair cells and afferent terminals, we systematically examined the distribution of muscarinic receptors in the vestibular periphery. The remainder of the paper summarizes that effort. First, we cloned and sequenced muscarinic receptor subtypes M2–M5 from pigeon. We were unable to clone M1. Fig. 3A shows the protein sequence corresponding to the transmembrane portion of the receptors. Note that there is considerable identity between the pigeon receptor subtypes and between pigeon M5 and human M5. Fig. 3B shows the third inner loop of the protein. It can be seen that there is considerable diversity in the amino acids in the i_3 loop. This region contains open reading frame of muscarinic receptor subtypes M2–M5 for pigeon. As a reference, the sequence for muscarinic receptor subtype M5 from human is also shown. The ruler and residue numbers are based on the human M5 sequence Genbank accession # NP_036257 (Bonner et al., 1988). The pigeon sequences are #s AY838767, AY838768, AY838766 and DQ357059 for the M2, M3, M4 and M5, respectively.

Two features are evident from Fig. 3. First, there is good identity between pigeon mACh receptor subtypes and between these subtypes and human M5 for transmembrane segments I–VII, inner and outer loops 1–2 and outer loop 3, but there is considerable heterogeneity for the inner loop 3. This large loop (227 amino acids) contains amino acids that are involved in G protein activation and coupling. Second, although the number of residues found inside and outside inner loop 3 are the same order of magnitude, there are 5.3 times (16/3) the number of serine/threonine residues that have a 50% chance of being a phosphorylation site on inner loop, i_3 .

Table 2 presents sequence identities for human muscarinic receptor subtypes M2–M5 compared to pigeon muscarinic receptor subtypes M2–M5. A comparison of probabilities indicates a novel result that human and pigeon muscarinic receptors of the same subtype have greater sequence identity than a comparison of the subtypes for the same species. A phylogenetic analysis of the comparisons shown in Table 2 (data not shown) confirms this conclusion.

Frozen section immunohistochemistry

Negative controls—We observed no significant autofluorescence (Del Castillo et al., 1989) in our tissue. We omitted primary or secondary antibodies when performing IH, IC and WB. These controls produced negative results. We pre-absorbed the antibody for each receptor

subtype with a blocking peptide or a purified protein (in the case of M4). Compared to the non pre-absorbed condition, the labeling of receptor subtypes on structures in the neuroepithelia were either partially or completely blocked (data not shown).

Positive controls—It has been reported that several mAChR subtypes are expressed in rat hippocampus (Van der Zee and Luiten, 1999; Rouse and Levey, 1997; Rouse and Levey, 1996; Hajos et al., 1998). Positive control experiments labeled areas of gerbil and rat hippocampus and striatum previously shown to express certain muscarinic receptor subtypes. Our results produced the same staining pattern as previous studies done with the monoclonal antibody M35 (Van der Zee and Luiten, 1999). The anti-M3 labeled the neuropil and produced same immuno-reactivity as described in a second publication (Levey et al., 1995) (data not shown). Our positive and negative control studies indicate that the antibodies we used for mAChR receptor subtypes are selective and specific.

Muscarinic receptor subtype expression in the vestibular periphery—All five mAChR subtypes were expressed in the pigeon's ampullary and utricular neuroepithelia, nerve fibers and ganglia as shown in Fig. 4. M1, M2, M3, M4 and M5 mAChR subtypes were expressed on different structures (such as supporting cells, hair cells, nerve fibers, ganglion cells, etc.), but in many cases they were co-expressed on the same element (e.g., M1–M5 on hair cells). There was no preferential expression in any region of semicircular canal crista (zones 1, 2 or 3) or utricular macula (S or ES regions).

Subtype M1 M1 was expressed in both types (type I and type II) of hair cells in the pigeon's crista and macula. Fig. 4 A-1 and A-2 illustrate double labeling of M1 and M4 in the crista (zone 3) and in the macula (striolar region). Type II hair cells are shown labeled in zone 3 (A-1) and type I hair cells, found primarily in zone 2, are not shown. However, type I hair cells, found in the striolar region of the utricular macula, are shown labeled in Fig. 4 A-2. Staining of the cuticular plates and cilia were found on the top of some hair cells. The supporting cells, which have their nuclei located near the basement membrane, were also labeled by M1. Fig. 4 A-1 and A-2 also show that M1 and M4 are co-localized inside nerve fibers and nerve terminals. In some areas, this co-localization can be seen as yellow structures. But in other cases M1 labeling appears to surround nerve fibers where there is no M4 expression. Since the nerve fibers are myelinated after they exit the basement membrane, the M1 labeling could indicate expression on myelin sheaths. In the vestibular ganglion (A-3), both M1 and M4 were expressed on both ganglion cells and nerve fibers. As in panels A1–2, M1 but not M4 was also expressed on tissue surrounding the ganglion cells and nerve fibers, which could be Schwann cells and/or myelin sheaths.

Subtype M2 M2 was expressed on hair cells and supporting cells in the ampullary (zone 2/3) and utricular neuroepithelium (Fig. 4 B-1, B-2). On some hair cells staining was present on the cuticular plate. M2 and M4 were co-expressed in the ganglion cells (Fig. 4 B-3). Their expression levels were not always uniform. Since the signal level of M2 staining was weak compared with M4, it was hard to identify M2/M4 co-localization in the nerve fibers and nerve terminals. However, IH using DAB indicated that M2 was expressed on nerve fibers and nerve terminals (see Fig. 5).

Subtype M3 M3 labeling are shown in Fig. 4C. In the crista (zone 2/3) and the macula striolar region of the utricle (Fig. 4 C-1 and C-2), M3 was expressed on hair cells especially at the super-nuclear regions and on the cuticular plates but supporting cells were negligibly stained, if at all. M3 labeled green parallel lines stopped just beneath the neuroepithelium basement membrane where the dendritic axons (nerve fibers) are known to lose their myelin. In contrast, M4 labeled nerve fibers extended into the neuroepithelium and terminated on hair cells. In C-3,

M3 is shown to be mainly located on the ganglion cell bodies. No obvious co-expression of M3 with M4 was observed in neural processes of the ganglion cells.

Subtype M4 The M4 receptor subtype was expressed on myelinated vestibular nerve fibers and unmyelinated nerve terminals in the neuroepithelia. M4 can be seen on the boutons en passant and calyces as well as the myelinated nerve fibers in zone 2/3 of semicircular canal crista (Fig. 4 D-1). The same labeling can be seen for the striolar zone of the utricular macula (Fig. 4 D-2). M4 was also expressed on vestibular ganglion cells as well as their dendrites and axons. No M4 labeling was seen on Schwann cells and myelin sheaths (Fig. 4 D-3).

Subtype M5 Structures labeled by M5 antibodies were very similar to those labeled by M1. M5 was expressed on both types of hair cells and supporting cells, as shown in the cristae (zone 3; Fig. 4 E-1) and utricular macular striolar region (Fig. 4 E-2). M5 was also expressed on accessory structures such as the cuticular plate and cilia. M5, with M4, was co-expressed on nerve fibers and nerve terminals. Additionally, M5 co-localized with M4 on some ganglion somata and their neural processes. Finally, M5 was expressed on putative Schwann cells (Fig. 4 E-3).

Muscarinic receptor subtype expression on myelin sheaths and Schwann cells

—Pigeon vestibular nerve fibers and ganglion cells are myelinated (Landolt et al., 1975; Landolt et al., 1973). To verify that some of the mAChR subtypes were expressed on myelin sheaths and Schwann cells of the peripheral vestibular nerve fibers, we used osmium tetroxide to stain myelin and compared this label to the DAB immuno-reactivity of mAChR subtypes in both vestibular end organs and Scarpa's ganglion (Fig. 5). Sections through the utricular macula had the same staining patterns as the semicircular canal crista and are not shown.

The data summarized in Fig. 5 indicates that M2 and M4 are extensively expressed on ganglion cell bodies and nerve fibers but not on myelin sheaths or Schwann cells (Fig. 5 B-2, B-4). On the other hand, M1, M3 and M5 subtypes are expressed on ganglion cells and both myelin sheaths and Schwann cells (Fig. 5 B-1, B-3 and B-5). In addition, all five subtypes stained nerve fibers, as shown in (A) the ampulla end organ region and (B) the Scarpa's ganglion area. The results of osmium tetroxide and DAB staining in vestibular ganglion and peripheral end organs (crista and macula) are consistent with the fluorescent IH results.

Isolated cell immunocytochemistry

Dissociated hair cell and ganglion cell IC was used to confirm the results of tissue IH and to examine expression of the receptor subtypes on fine structures such as the cuticular plate and cilia.

Both types of hair cells (type I and type II) from both the crista and utricle were examined. No difference in staining patterns was found in the hair cells from either the crista or the utricle for each mAChR subtype. Type I and type II hair cells can be distinguished by the ratio of the neck width to cuticular plate width and the neck width to the body width (Ricci et al., 1997). All five mAChR subtypes were expressed on both types of hair cells and ganglion cells (Fig. 6). However, there was differential expression of the subtypes on certain cellular structures, including the cilia and cuticular plates. Only M1 and M5 were expressed on the cilia while only M4 was not expressed on the cuticular plates.

M4 and M5 appear expressed mainly in the hair cell membrane, as expected. However, for the other subtypes mAChR labeling was found to be in the cell cytoplasm or in both the cytoplasm and plasmalemma. In no case, however, did the nucleus of the cells show label.

Table 3 summarizes the results derived from all the IH and IC studies using frozen sections and dissociated cells.

Western blotting

The results of the Western blotting studies presented below are consistent with the data presented in Table 3 above. The results for each mAChR are summarized in turn.

Subtype M1—Multiple bands were seen for all three lysates (VG, NE and NF) but the M1 negative control (H⁻) showed a partial blockage of the band corresponding to a molecular mass (Mr) of 57 kDa compared with the normal control (H) (Fig. 7). At this position, each lysate showed the same protein band. The molecular mass for pigeon M1 is unknown but in human, the molecular mass of M1 is estimated to be 51 kDa.

Subtype M2—Consistent with the results of frozen section staining, M2 showed weaker signals for protein samples from VG and NF compared to NE (Fig. 7). A protein band at 47 kDa was completely blocked with pre-adsorption. This value is close to the theoretical Mr of pigeon M2 protein which should be 51.4 kDa.

Subtype M3—We located two protein bands that were blocked using pre-absorption controls (Fig. 7). One protein band was located at 73 kDa; the other band was at 48 kDa. Two protein bands shown in Fig. 7 could be due to the breakage of proteins during sample preparation. This result is identical to the blot shown on the manufacturer's data sheet. The inhibition of these two bands by the negative control antigen indicates that both bands are related to M3 protein (personal communication, Ofra Gohar, Ph.D, Alomone Labs, Ltd., Jerusalem, Israel). The Mr of pigeon M3 should be 72 kDa. At that Mr M3 was present in all three vestibular samples, but weak in the nerve fibers which was consistent with the IH fluorescent and DAB results.

Subtype M4—Only one protein band for M4 was clearly seen on the VG, NE and NF lanes (Fig. 7). No matter whether we increased the boiling time for denaturing the sample proteins or used other protein samples, such as gerbil's heart muscle, striatum or hippocampus, a single band for M4 protein was always located at 98 kDa (data not shown). However, the theoretical Mr of M4 from pigeon is 55 kDa. This difference in molecular mass may be the result of post-translational modification (see Fig. 3) resulting in the increased molecular mass. Compared with the normal control (H), the M4 protein band was partially blocked in the pre-adsorption control (H⁻) indicating that this protein band was indeed M4.

Subtype M5—M5 control lanes had at least three protein bands, only the one located at 74 kDa could be blocked with excess antigen. This product was present in the VG and NE lanes but barely detectable for NF. The measured Mr of M5 is 74 kDa which is larger than that calculated from its gene sequence (59.9 kDa). Again this could be due to the post-translational modification (Note the numerous glycosylation and phosphorylation sites in the sequences presented in Fig. 3).

DISCUSSION

Patch clamp recordings from pigeon native hair cells show that carbachol, a cholinergic agonist, reduces the current through the inward rectifier pKir2.1 channel. Previously, we presented preliminary results (Li and Correia, 2005) that carbachol also reduced the current in tsA-201 cells transfected with a vector that included M3, EGFP and pKir2.1. The reduction in pKir2.1 current leads to a depolarization of the cell membrane and therefore modulates the cell's sensitivity (Correia et al., 2004). This important physiological function prompted us to seek

the source of ACh in the pigeon vestibular neuroepithelium and describe the muscarinic receptor subtypes.

It has long been known that in mammals cholinergic synapses exist between efferent vestibular neurons, hair cells and afferent terminals (Gacek et al., 1965; Iurato et al., 1971). Moreover, it has been shown that, like mammals (Goldberg and Fernandez, 1980), the cell bodies of putative avian efferent neurons are present in the brainstem (Eden and Correia, 1982; Schwarz et al., 1981). Cholinergic synapses exist in the pigeon vestibular neuroepithelium with anatomical features (See Fig. 2) like those seen for efferent synapses in mammals (Wersall, 1956). That is, there is a vesiculated pre-synaptic bouton (efferent terminal), a pre-synaptic electron density (or a synaptic cleft) and a post-synaptic cistern. Analysis of a sample of 44 synapses reveals that efferent boutons synapse on hair cells, both type I and type II as well as calyx and bouton afferent endings. The distribution of types of synapses show the synapses between type II hair cells and efferents are the most frequent whereas synapses between type I hair cells and efferents are the least frequent. However, the novel finding of a synaptic contact between a type I hair cell and an efferent terminal indicates that efferents can control type I hair cells via either the cell body and/or the calyx.

To determine the molecular structure of pigeon mAChRs we cloned and sequenced M2-M5. We aligned the 4 pigeon mAChR sequences with human M5 and noticed that in both pigeon (M2-M5) and human M5 in all domains of the protein (Fig. 3A), except in the inner loop 3 domain (Fig. 3B), there is good homology. The i_3 loop domain in each subtype has considerable diversity. In addition, there is greater identity between comparable subtype isoforms in pigeon compared to man than between the 5 subtypes in each species (Table 2).

The vast majority of proteins are subject to one or a variety of post-translational modifications which are functionally relevant. The mAChRs can be modified at glycosylation sites near the start codon of the ORF (Fig. 3A) or/and at phosphorylation sites in the inner loop 3 (Fig. 3B). It has been shown that protein glycosylation is required for transport of the mAChR to the cell surface (Liles et al., 1986), but these sites are not involved in cell surface localization or ligand binding (Van Koppen and Nathanson, 1990). The rich S/T i_3 domain in M1-M3 has been demonstrated to be responsible for mAChR internalization/sequestration leading to a reduction in binding sites (Lameh et al., 1992; Moro et al., 1993). Moreover, it has been suggested that phosphorylation of members of the mAChR family may underlie uncoupling of receptors from G-proteins and may be responsible for the rapid phase of receptor desensitization (Hosey et al., 1995; Contrera et al., 1993).

We obtained very consistent results using 3 different methods (IH, IC and WB) to identify the mAChRs on neural and accessory structures in the pigeon vestibular periphery. A schematic summarizing those results and indicating the distribution of mAChR subtype expression in the pigeon's vestibular neuroepithelia, afferent fibers, ganglion cells and accessory elements is presented in Fig. 8. Our results do not speak to the amount of expression of M1-M5 but do indicate that mAChRs are widely present and co-expressed on vestibular peripheral system. Furthermore, we cannot dismiss the additional possibility of mAChR expression on efferent fibers and terminals forming autoreceptors. This possibility was not explored in this study because of the difficulty of separating afferent and efferent terminals morphologically at the light microscopic level.

The possible roles of the mAChR subtypes in the vestibular periphery may be surmised based on knowledge gained from studies of mAChRs in other physiological systems. In general, it is known that acetylcholine receptors serve autocrine and neuronal functions (Wessler et al., 1998). The autocrine functions include control of cell growth and proliferation and control of the release of chemical mediators. (Fujii and Kawashima, 2001; Chi et al., 2002; Fritz et al.,

1999;Nomura et al., 2003;Kawashima and Fujii, 2004). These functions could obviously be important in hair cell regeneration or maintenance and replenishment of endolymph and perilymph (Wangemann et al., 2001). The expression of mAChR subtypes on supporting cells and Schwann cell may provide a non-neuronal cholinergic function (Eglen, 2006). However, the abundance of mAChRs on the neuronal elements in the peripheral labyrinth strongly suggests mAChR involvement in sensory transduction and signal modulation.

The hair cell cilia and the underlying cuticular plate are crucial structures for sensory transduction. The cilia consist of multiple stereocilia and a single kinocilium. Stereocilia possess mechanically gated channels (Hudspeth, 1989) while the kinocilium serves as a linkage to transfer the mechanical stimuli to the stereocilia (Hudspeth and Jacobs, 1979). In this study we determined that cilia, at least stereocilia, showed clear expression for the odd-numbered mAChR subtypes: M1 and M5. The major component of the stereocilia is actin (Flock and Cheung, 1977; Tilney et al., 1980; Shepherd et al., 1989), which is cross-linked by another filament protein fimbrin (Hofer et al., 1997; Drenckhahn et al., 1991; Tilney et al., 1989; Flock et al., 1982). Stereocilia have a similar structure and protein composition to microvilli of the intestine (Mooseker and Tilney, 1975). The rootlets of microvilli are enmeshed in the terminal web which has fine filamentous networks of actin-associated cross-linkers, such as myosin, tropomyosin, fodrin and spectrin-like proteins (Glenney, Jr. et al., 1982; Glenney, Jr. et al., 1983; Burgess et al., 1987; Mooseker et al., 1978; Citi and Kendrick-Jones, 1991). Stereocilia are embedded in the cuticular plates which contain actin, alpha-actin, myosin, tropomyosin, fodrin, fimbrin, etc. (Slepecky and Chamberlain, 1985; Hasson et al., 1997; Sobin and Flock, 1983; Scarfone et al., 1988). When acetylcholine is applied to ciliated epithelial cells, the ciliary beat frequency (CBF) is increased in cultured ovine tracheal epithelial cells (Salathe and Bookman, 1995), cultured frog esophagus epithelium (Zagoory et al., 2001), cultured explants of *Rana pipiens* palate (Aiello et al., 1991) and cultured adenoid explants (Yang and McCaffrey, 1996). Selective M1 and M3 mAChR subtype antagonists significantly inhibited the ACh induced stimulation of CBF. A similar role for acetylcholine in hair cell stereocilia could obtain.

In hair cells, the movement of cuticular plate and stereociliary bundle play a role in hair cell motile responses (Zenner, 1988). Furthermore, the control of hair cell motile responses may due to the phosphoinositide metabolism (Schacht J. and Zenner H.P., 1986; Schacht and Zenner, 1987), which changes the intracellular calcium level that in turn modulates actin polymerization and microfilament-based motility (Lassing and Lindberg, 1988). In rat cochlear hair cells, the activation of M3 is thought to increase the inositol phosphate cascade (Guiramand et al., 1990). Considering the intracellular signaling pathway and the molecules responsible for the modulation of ciliated epithelium movements, mAChR activation could regulate the mechano-electrical transduction of vestibular hair cells at least at three places: stereocilia, cuticular plate and hair cell body. The expression of mAChR subtypes M1 and M5 on cilia, M1, M2, M3 and M5 on cuticular plates and hair cell bodies and M4 on the hair cell body indicates that each mAChR subtype might play different roles to regulate the hair bundle sensory transduction and signal processing in the hair cell. Recent studies of hair cells indicate that hair bundle motility can occur spontaneously or be evoked (Martin and Hudspeth, 1999). Thus, the wide expression of mAChR on the vestibular hair bundle, cuticular plate and hair cell body could exercise regulatory control of hair bundle spontaneous or driven motion and consequently improve or degrade (modulate) the efficiency of hair cell transduction. An interesting and perplexing question, however, is where are the ACh stores located that activate receptors on the cilia and cuticular plates? The cilia and cuticular plates are bordered by endolymph. They are separated from the neuroepithelium by the tight junctions between hair cells and adjacent supporting cells. It could be suggested that hair cells excrete ACh through the apical surface of the cuticular plate but this is unproven and needs future study.

The expression of all five mAChR subtypes on vestibular hair cells indicates that they probably play an important role in modulating ion channel activity involved in changing the hair cell membrane potential and regulating transmitter release from hair cells onto afferent terminals. The signaling molecule for this sequence of events would be the release of ACh by efferent terminals onto hair cells and nerve terminals. In isolated frog vestibular hair cells, application of the mAChR agonist carbachol stimulated the transmitter release from hair cells (Derbenev et al., 2005). Notably, carbachol decreases currents in Kir2.1 channels (Jones et al., 1991; Firth and Jones, 2001; Pemberton and Jones, 1997; Jones, 1996) and decreases currents in KCNQ channels (Selyanko et al., 2000; Shen et al., 2005; Wei et al., 2005) and either increases (M4 acting on AtT-20 cells) or decreases (M1–M5 acting on NIH3T3 transfected cells) current in calcium L type channels (Pemberton and Jones, 1995; Pemberton and Jones, 1997). The above mentioned channels whose currents can be modulated by carbachol have been identified as being expressed on vestibular type II hair cells. These channels include Kir2.1 (Correia et al., 2004), putative KCNQ (Rennie et al., 2001) and L type Ca^{2+} channels (Lang and Correia, 1989).

Different AChR subtypes apparently modulate different ion channels in different polarizing directions. For example, the odd-number group of mAChRs, mediated through *Gaq/11* protein, can produce inhibitory effects on KCNQ and Kir2.1 channels and depolarize the hair cell membrane potential. On the other hand, it has been found that the cochlear hair cell membrane potential can be hyperpolarized by activating the Ca^{2+} activated K^{+} channel (Shigemoto and Ohmori, 1991). The even-numbered but not odd-numbered mAChRs, are responsible for increasing the conductance of the Ca^{2+} activated K^{+} channel (Kong et al., 2005). Again, Ca^{2+} activated K^{+} channels are present on pigeon vestibular hair cells (Lang and Correia, 1989).

Supporting cells in the pigeon vestibular neuroepithelium express Kir2.1 ion channels (Masetto and Correia, 1997a) and show M1, M2, M3 and M5 expression. They could communicate with hair cells through gap junctions and be modulated by ACh. However, the question again arises - where does the ACh transmitter come from? We were unable to visualize efferent terminal-supporting cell synapses, using either light or TEM microscopy. There are only suggestions of such synapses. For example, electron microscopic analyses have demonstrated that labeled immature efferent axons were present among supporting cells of the greater epithelial ridge at P0 in the rat cochlea (Bruce et al., 2000) and in mouse cochlea efferents have extensive collaterals among supporting cells of the greater epithelial ridge from 16.5 days post coitum onwards (Bruce et al., 1997).

Scarpa's ganglion bipolar cells and their neural processes express all the mAChR subtypes. This result is consistent with earlier studies of mAChR message. Using RT-PCR mRNA analysis, rat vestibular peripheral end organ and Scarpa's ganglion cells expressed message for all five mAChR subtypes while in human message for only M1, M2 and M5 was found (Ishiyama et al., 1997; Wackym et al., 1996). The expression of muscarinic receptors on vestibular ganglion cells and nerve fibers could be involved in the regulation of the axoplasmic transport (Kawakami et al., 1995), neurofilament expression and neurite growth (Small et al., 1995).

Vestibular ganglion cells and nerve fibers are enveloped by myelin sheaths formed by neuroglia, Schwann cells, (Kido et al., 1993; Kido and Sekitani, 1994; Ona, 1993; Samandari and Bogusch, 1981; Landolt et al., 1975; Landolt et al., 1973). Previous studies (Torskaia and Goloborodko, 1977; Haynes, 1983; Lan et al., 1996) have reported that glial cells demonstrate the presence of acetylcholinesterase and choline acetyltransferase which might indicate the existence of cholinergic receptors on the glial cells. Our results show that M1, M3 and M5 are expressed on both myelin sheaths and putative Schwann cells. It is known that K^{+} channels

play an important role in the formation, maintenance and proliferation of myelin (Sobko et al., 1998; Wilson and Chiu, 1990). M1, M3 and M5 may modulate K⁺ ion channels in myelin and Schwann cells and therefore may assist in the initiation, repair and proliferation of myelin. Moreover, the activation of the intracellular signal effectors, such as mitogen-activated protein kinase might also participate in the controlling of cell growth and proliferation (Hamilton and Nathanson, 2001; Berkeley and Levey, 2000).

Finally, the mAChR subtypes that were grouped on different vestibular peripheral structures were not always the same (See legend of Fig. 8). A similar heterogeneity of muscarinic receptor subtype co-expression has been found in other tissues e.g., salivary gland, ileum, lung and neuromuscular junctions (Levey, 1993; Dorje et al., 1991; Garcia et al., 2005). Similarities and differences also exist between mAChR subtype expression in the pigeon vestibular periphery and the rat cochlea. For example, the odd numbered mAChRs are expressed on hair cells, supporting cells and ganglion cells in both systems. But, while M2 and M4 subtypes are expressed on both types of hair cells, nerve fibers (particularly M4) and ganglion cells in the peripheral vestibular system, their expression was not detected in cochlea (Khan et al., 2002).

In conclusion, based on previous work in other tissues and cell types, we have suggested possible roles of the mAChRs expressed in the pigeon vestibular periphery. The expression of nAChRs in the vestibular periphery is beyond the scope of the present paper. The exciting challenge for future research will be to decipher the functional contribution of each mAChR subtype or groups of subtypes on each of the neural structures where we have found their expression. A second challenge will be the parceling out of the role of nicotinic receptor subtypes from those of muscarinic subtypes and vice versa in the vestibular periphery. To do this we will need much more specific mAChR and nAChR agonists and antagonists and robust supplementary electrophysiological techniques.

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LIST OF ABBREVIATIONS

AB	afferent bouton
Ab	antibody
Ag	antigen
C	calyx
CP	cuticular plate
EGFP	

	enhanced green fluorescent protein
ES	extrastrilar zone
ET	each time
DAB	diaminobenzidine
GC	ganglion cell
HC I	type I hair cell
HC II	type II hair cell
HM2-5	human muscarinic receptor subtype 2-5
HRP	horseradish peroxidase
IC	immunocytochemistry
ID	internal diameter
IH	immunohistochemistry
M	myeline sheath
mAChRs	muscarinic acetylcholine receptors
Mr	molecular mass
nAChR	nicotinic acetylcholine receptor
NE	neuroepithelium
NF	nerve fiber
OD	outside diameter
ORF	open reading frame

OsO₄	osmium tetroxide
pKir2.1	pigeon's inwardly rectifying potassium channel
PBS	phosphate-buffered saline
RIPA	radioimmunoprecipitation
PM2-5	pigeon muscarinic receptor subtype 2-5
PSC	post synaptic cistern
PSD	pre-synaptic density
RT	room temperature
SB	synaptic body
SC	supporting cell
SWC	Schwann cell
TBST	Tris-Buffered Saline Tween-20 solution
WB	Western blotting
VB	vesiculated bouton
VG	vestibular ganglion

Fig 1.

Traces of inward and outward currents produced by inward rectifier K^+ channels (Kir2.1) in a pigeon type II vestibular hair cell found in an epithelial slice. Traces are shown for the control (Aa), carbachol application (Ab) and recovery (Ac). As seen in the voltage protocol traces (Ad) the cell was held at -60 mV and voltage was varied in 10 mV increments from -120 to -40 mV. (B) An I/V plot showing the results of control (■), carbachol (●) and recovery (▲) responses.

Fig 2.

Efferent synapses on type I hair cell (A); type II hair cell; (B); afferent nerve fiber (C) and type I hair cell calyx (D). Inset in panel A shows enlarged view of synaptic contact between efferent terminal and type I hair cell. Shown is a cuticular plate (CP); post synaptic cistern (PSC); vesiculated bouton (VB-efferent); type I hair cell (HC I); a calyx (C); a type II hair cell (HC II); a synaptic body (SB); afferent bouton (AB) and pre-synaptic density (PSD). Bars in: inset in A = 20 nm; in Fig. A = 5 μm; in B–D = 200 nm.

Fig 3. (A) Alignment of human M5 (HM5) with pigeon M2–M5 (PM2–PM5). Shown are all seven transmembrane segments and outer and inner segments 1–2. (B) Alignment of HM5 and PM2–

PM5. Shown is the inner loop 3. This segment is believed to contain three amino acids (K339, A440 and A441) that are involved in G protein activation and coupling (Burstein et al., 1998). In the alignment in both A and B only identical residues are shown. The seven transmembrane segments are bracketed and labeled by roman numerals. The three inner and outer segments are labeled **i**₁₋₃ and **o**₁₋₃. Within the brackets, the range of residues is given. Serine, threonine and tyrosine residues with a probability of $P > 0.5$ of being a phosphorylation site are labeled with #, + and Δ, respectively. Glycosylation sites are identified by *. The amino and carboxyl terminals are identified by H₂N and COOH, respectively.

Fig 4.

Fluorescent labeling of mAChR subtype expression on vestibular peripheral structures in the crista, utricle and ganglion. The results shown here are the anti-M4 antibody reacted with a red fluorescent secondary antibody while other mAChR subtypes reacted with a green secondary antibody. No differences were found when the red/green fluorescent secondary antibodies were exchanged. The co-expression of M4 with M1, M3 and M5 on nerve fibers, nerve terminals and ganglion cells are marked by arrows. Myelin sheaths and Schwann cells surrounding ganglion cells are pointed at by single arrowheads. Cuticular plates w/ or w/o protruding cilia are pointed at by double arrows. The labeling above the row of hair cells in utricle (especially

in E2) is the artifact induced by labeling the otoconial membrane which was not removed.
Scale bars = 10 μ m.

Fig 5. DAB staining of (A) semicircular canal crista and (B) vestibular ganglion. The number in each panel corresponds to the mAChR subtype number. Myelin sheaths and Schwann cells circumscribe the nerve fibers and ganglion cells (black arrows). Nerve fibers expressing M1, M2, M4 and M5 traverse the basement membrane and terminate in the neuroepithelium (arrowheads). There is no M3 expression on peripheral nerve terminals inside of the neuroepithelium. The myelin sheath staining stops just beneath the basement membrane (double arrowheads in A-3). Scale bars = 10 μ m.

Fig 6.

Isolated type I and type II hair cells from ampulla and utricle, as well as vestibular ganglion cells labeled with each of five primary antibodies are shown in the column. The primary antibodies were labeled with a green-tagged secondary antibody. Corresponding differential interference contrast photomicrographs are shown in adjacent columns. Note that M1 and M5 are located on hair cells, cilia and cuticular plates; M2 and M3 are shown on hair cells and cuticular plates and M4 is expressed only on hair cells. All five mAChRs are expressed on ganglion cells (column 3). Scale bars = 5 μ m.

Fig 7.

Western blotting results for excised vestibular ganglion (VG), dissociated vestibular end organ epithelium (NE) and dissociated nerve fibers (NF). Protein samples used for the normal controls (H) and pre-adsorption controls (H-) were from the pigeon's hippocampus. Molecular mass based on markers are indicated on the left. The measured and theoretical molecular masses (in parentheses) are shown on the right. Non stringent "Dirty" Western blot techniques were applied to help identify all mAChR subtype protein products. Molecular mass of M1 mAChR in human is marked by *.

Fig 8.

Summary of M1–M5 mAChR subtype expression in the pigeon vestibular periphery. Shown are the two types (I and II) of hair cells (light green); cuticular plates (orange); cilia (violet); supporting cells (SC, blue); afferent endings (calyxes and boutons, red); nerve fibers of ganglion cells and ganglion cells (GC, light green); myelin sheaths (M) and Schwann cells (SWC, dark green). Each color indicates the location of expression and the mAChR subtypes that were expressed. Although efferent fibers and efferent terminals (yellow) are shown for reference no attempt was made to determine mAChR subtype expression on these structures because of the difficulty of separating afferent and efferent terminals at the light microscopic level.

Table 1

Antibodies and Antigens Used for Muscarinic Receptor Subtype Studies

[[mACHR Subtypes]]	[[Item]] *	[[Cat. #]]	[[Host]]	[[Source]]	[[Company]]	[[Epitope]]	[[Identity to Pigeon]]	[[Pigeon Accession #]]
[[mACHR M1]]	[[Ab]]	[[Q4A173R]]	[[Rabbit]]	[[Human]]	[[Biodesign]]	[[Amino acid]]	[[---]]	[[---]]
[[mACHR M2]]	[[Ag]]	[[A4A730H]]	[[Human]]	[[Human]]	[[International]]	[[451-460]]	[[87/132 (65.9%)]]	[[AY838767]]
	[[Ab]]	[[AMR-002]]	[[Rabbit]]	[[Human]]	[[Alomone Labs]]	[[Amino acid]]	[[17/19 (89.5%)]]	[[AY838768]]
[[mACHR M3]]	[[Ag]]	[[For AMR-002]]	[[Human]]	[[Human]]	[[Alomone Labs]]	[[225-356]]	[[101/194 (52.1%)]]	[[AY838766]]
	[[Ab]]	[[AMR-006]]	[[Rabbit]]	[[Human]]	[[Alomone Labs]]	[[Amino acid]]	[[101/194 (52.1%)]]	[[AY838766]]
[[mACHR M4]]	[[Ag]]	[[For AMR-006]]	[[Human]]	[[Rat]]	[[Chemicon]]	[[461-479]]	[[92/184 (50%)]]	
	[[Ab]]	[[MAB1578]]	[[Mouse]]	[[Human]]	[[International]]	[[217-401]]	[[13/16 (81.3%)]]	[[DQ357059]]
[[mACHR M5]]	[[Protein]]	[[sc-4505 WB]]	[[Human]]	[[Human]]	[[Biotechnology]]	[[220-394]]	[[13/16 (81.3%)]]	[[DQ357059]]
	[[Ab]]	[[Q4A871R]]	[[Rabbit]]	[[Human]]	[[Biodesign]]	[[Amino acid]]	[[13/16 (81.3%)]]	[[DQ357059]]
	[[Ag]]	[[A4A738H]]	[[Human]]	[[Human]]	[[International]]	[[516-531]]	[[13/16 (81.3%)]]	[[DQ357059]]

* Ab, Antibody; Ag, Antigen.

Table 2

Sequence pair identities of Clustal W analysis comparing human and pigeon muscarinic subtypes M2–M5. Percent identity for the same subtype but across species appears bolded in the cells. Note that these are the highest values.

[[HM2]]	[[HM3]]	[[HM4]]	[[HM5]]	[[PM2]]	[[PM3]]	[[PM4]]	[[PM5]]	[[[]]]
[[[]]]	[[46.5]]	[[59.0]]	[[43.9]]	87.3	[[46.3]]	[[63.4]]	[[44.5]]	[[HM2]]
[[[]]]	[[[]]]	[[39.4]]	[[47.9]]	[[37.4]]	80.3	[[37.9]]	[[49.3]]	[[HM3]]
[[[]]]	[[[]]]	[[[]]]	[[43.8]]	[[57.8]]	[[45.3]]	75.4	[[44.1]]	[[HM4]]
[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[39.1]]	[[51.7]]	[[40.6]]	74.8	[[HM5]]
[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[45.3]]	[[63.3]]	[[39.5]]	[[PM2]]
[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[34.1]]	[[54.4]]	[[PM3]]
[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[41.0]]	[[PM4]]
[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[[]]]	[[PM5]]

Table 3
Summary of mAChR subtype expression on the peripheral vestibular neurons

[[[]]]	[[M1]]	[[M2]]	[[M3]]	[[M4]]	[[M5]]
[[Hair Cells]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]
[[Cilia]]	[[Yes]]	[[No]]	[[No]]	[[No]]	[[Yes]]
[[Cuticular plate]]	[[Yes]]	[[Yes]]	[[Yes]]	[[No]]	[[Yes]]
[[Supporting cells]]	[[Yes]]	[[Yes]]	[[No]]	[[No]]	[[Yes]]
[[Nerve Terminals]]	[[Yes]]	[[Yes]]	[[No]]	[[Yes]]	[[Yes]]
[[Nerve Fibers]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]
[[Myelin Sheath]]	[[Yes]]	[[No]]	[[Yes]]	[[No]]	[[Yes]]
[[Ganglion Cells]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]	[[Yes]]
[[Schwann Cells]]	[[Yes]]	[[No]]	[[Yes]]	[[No]]	[[Yes]]