Neurotransmitter and neurotransmitter receptor expression in the saccule of the human vestibular system

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ABSTRACT

The saccule is one of the vestibular sensory organs of the inner ear. It detects head movements and provides information to maintain balance and orient in space. Despite its critical role, very little is known about its neurotransmission and regulation. Multiple disease entities and medications affect balance, which is why information on neurotransmission in the vestibular end organs including the saccule could have important pharmacological implications. To the best of our knowledge, this is the first paper to describe immunohistochemical expression of a large panel of neurotransmitters and receptors in the human saccule. Saccular tissue was sampled freshly during surgery. Based partly on previous findings in non-humans and partly on potential biological relevance, the neurotransmitters cholecystokinin, dopamine, GABA, glutamate, histamine and serotonin as well as receptors for these were selected for the tested panel. The neuroepithelium expressed glutamate receptor 1 (GluR1), metabotropic glutamate receptor (mGluR), GABA A receptor α, GABA B receptor 2 and cholecystokinin receptor B (CCKBR), whereas α-glutamate, GluR1, CCKBR, GABA α, dopamine and serotonin receptor 1D were expressed in the subepithelial stroma. The non-sensory epithelium expressed GluR1, mGluR, histamine receptor 3, CCKAR and dopamine transporter. These findings provide a basis for pharmacological research and potential drug development.

1. Introduction

The saccule is part of the inner ear vestibular system and plays an important role for balance and orientation in space. Together with the utricle, it is sensitive to linear acceleration (Kingma and van de Berg, 2016; Zaleski-King et al., 2019). Fig. 1 illustrates the inner ear and the inner ear vestibular system.

The sensory neuroepithelial cells of the saccule are located in a single patch, the macula, with a more central striola and a peripheral extra-striola region (Kingma and van de Berg, 2016; Merchant et al., 2010; Zaleski-King et al., 2019). The non-sensory part of the saccule is lined by a single layer of flat to cubic epithelium. The sensory cells are hair cells that function as mechanoreceptors (Kingma and van de Berg, 2016; Zaleski-King et al., 2019). There are two types of vestibular hair cells: type I and type II, and three types of afferent nerve fibers (Balaban, 2016; Merchant et al., 2010; Zaleski-King et al., 2019). Supporting cells surround the hair cells (Engström et al., 1972; Smith, 1956; Taylor et al., 2015). Fig. 2 illustrates the sensory neuroepithelium of the saccular macula.

The afferent fibers are bipolar neurons with cell bodies located in the vestibular ganglion (Scarpa’s ganglion) in the internal auditory canal (Fig. 1) (Balaban, 2016; Merchant et al., 2010; Zaleski-King et al., 2019). Vestibular efferents synapse with the calyx of type I hair cells, as well as directly with the type II hair cells (Fig. 2) (Balaban, 2016; Merchant et al., 2010; Zaleski-King et al., 2019).

The neurotransmitter mechanism of the afferent calyx-type I and bouton-type II hair cell synapses is likely the same (Balaban, 2016). There is evidence from several studies that glutamate is the primary excitatory neurotransmitter (Table 1). Amongst other studies, Dememes et al., 1995 investigated the presence of glutamate receptors including glutamate receptor 1 (GluR1) in rat and guinea pig vestibular neuroepithelium. By using anti-body staining, light and electron microscopy,
they suggested that glutamate has a site of action both pre- and post-synaptically (Starr and Sewell, 1990).

Using postmortem human temporal bones Schrott-Fischer et al. observed GABA immunoreactivity on vesiculated nerve endings and on unmyelinated nerve fibers of the saccular macula (Schrott-Fischer et al., 2002). They did not observe GABA reactivity on hair cells, clyces or myelinated fibers. A similar electron microscopic study, this time in rat myelinated fibers. A similar electron microscopic study, this time in rat showed immunoreactive vesiculated nerve endings terminating on calyces of type I hair cells providing evidence that GABA may be involved in efferent transmission (Kong et al., 1998a, 1998b).

Only few other studies on human tissue have been conducted, of these a number of immunohistochemical studies on fresh human endolymphatic sac epithelium (Møller et al., 2016, 2017a). These studies demonstrated expression of cholecystokinin receptor A (CCKAR) and B (CCKBR), histamine receptor 3 (H3R), and serotonin receptor 1D (5-HT1D,R).

Multiple disease entities of the peripheral vestibular system may involve the saccule (Brandt et al., 2013; Mattingly et al., 2019). Knowledge on neurotransmission therefore have pathophysiological and pharmacological implications: both in treating vestibular disorders and in understanding vestibular side effects of pharmacological treatment. Despite this, very little is known about neurotransmission in the vestibular system of the inner ear in humans.

This paper is, to our best knowledge, the first to describe expression of neurotransmitter and neurotransmitter receptors in the human saccule. We chose to investigate expression of a panel of neurotransmitters and neurotransmitter receptors in tissue sampled freshly during surgery. The molecules of the panel were selected partly on previous findings and partly on potential biological relevance, which is described in more detail in Table 1. The panel included the neurotransmitters cholecystokinin, dopamine, GABA, glutamate, histamine and serotonin as well as receptors for these.

2. Materials and methods

2.1. Tissue sampling

The saccule was sampled during surgery for vestibular schwannoma using the translabyrinthine approach in 12 consecutive patients (mean age 57 years, range 48–69). None of the tumors extended into the inner ear, thus all were located intracranially. During the translabyrinthine approach, the vestibular system of the inner ear (semicircular canals and vestibule) is opened and removed by drilling. Careful blue-lining and opening of the vestibule ensured that no damage was inflicted before removal of the saccule from the vestibule. Upon removal from the vestibule, the fresh tissue was immersed immediately in 3% Paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for hematoxylin-eosin (HE) staining and immunohistochemical analyses (Chan, 2014; Feldman and Wolfe, 2014). The study was approved by the Regional Research Ethics Committee (protocol: H-3–2011-105) and a written consent was obtained from all patients.

Tissue preparation and immunohistochemical staining methods are as described in previous papers of our group (Møller et al., 2015, 2017a), and are explained below.

2.2. Tissue preparation

After fixation, the tissue samples from the saccule were embedded in paraffin blocks, from which 5 μm sections were cut and mounted on glass slides. The slides were dried, then de-paraffinized with xylol, rehydrated in graded alcohols, and rinsed in distilled water.

To illustrate general histomorphology of freshly sampled tissue and to serve as a reference for the sections stained by immunohistochemistry, a few slides from each individual were stained with HE.

For the slides used for immunohistochemistry, antigen retrieval was accomplished by placing the slides in Coplin jars with either Tris-EGTA or citrate buffer, followed by heating for 6 min in a microwave (Tris-EGTA buffer: 10 mM Tris Base, 1 mM EDTA 0.05 % Tween-20, pH 9.0. Citrate buffer: 0.1 mol/L, pH 6.0) Some tissue sections were solely stored in Tris buffer, thus not exposed to antigen unmasking. All slides were rinsed in Tris buffered saline (TBS) and immersed in goat serum diluent for 15 min. Also, to prevent nonspecific staining, slides were preincubated in 1% bovine serum albumin in TBS, pH 7.4 for 1 h.

Fig. 1. Schematic of the inner ear.

The osseous labyrinth of the inner ear (green) consists of both the hearing organ, the cochlea, and the inner ear vestibular system comprising the vestibule and the three semicircular canals. Within the vestibule are the saccule and the utricle of the membranous labyrinth (blue), which play an important role for balance and orientation in space. The membranous labyrinth (blue) extends into the cochlea and the semicircular canals. Each of the canals has a bulbous enlargement at one end, the ampulla, comprising the sensory epithelium of the canal. The afferent fibers of the saccule run primarily in the inferior division of the vestibular nerve (yellow), but the superior division of the nerve (yellow) also carries some saccular afferent fibers. The cell bodies of these bipolar afferents lie in the vestibular Scarpa’s ganglion in the internal auditory canal. The saccule also receives efferent nerve fibers via the vestibular nerve. The orientation of the schematic is similar to Fig. 3A.
2.3. Immunohistochemical staining

The prepared sections were incubated with the specific antibody for 18 h at 4 °C using the two-step immunohistochemistry staining technique Dako EnVision + System-HRP (product number K4003 and 4007, Dako, Glostrup, Denmark). After incubation with the antibody, a tyramide signal amplification kit (Molecular Probes, Eugene, OR, USA) was used to improve detection of antibody binding. After incubation with the signal amplification kit, the sections were rinsed in TBS and stained for cellular nuclei with a 4,6-diamino-2-phenylindole-2HCl (DAPI) (Carlsbad, CA, USA); 0.2 mg was dissolved in 1 mL distilled water and 10 μL of this solution was mixed with 10 mL McIlvaine buffer, pH 7.0 (Moller et al., 2017b). The DAPI solution was applied to the slides and incubated 15 min in the dark, followed by mounting with Gel Mount (Sigma-Aldrich (G0918) St. Louis, MO, USA) and cover slips.

Negative controls comprised incubation A) without the relevant antibody; B) with an irrelevant antibody; C) without Dako EnVision + System-HRP; D) with the tyramide signal amplification kit alone; or E) with DAPI alone.

2.4. Neurotransmitter and neurotransmitter receptor antibodies

The primary antibodies included:

1) Cholecystokinin (CCK): Rabbit anti—CCK8 polyclonal antibody, Bioss, MA, USA; catalog no. bs-0764R (https://www.biossusa.com/). Source: Keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide derived from human CCK8. Purified by Protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Moller et al., 2017a).

2) Cholecystokinin receptor A (CCKAR): Rabbit anti—CCKAR polyclonal antibody, Bioss, MA, USA; catalog no. bs-11514R (https://www.biossusa.com/products/bs-11514r). Source: KLH-conjugated synthetic peptide derived from human CCKAR. Purified by protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Moller et al., 2017a).

3) Cholecystokinin receptor B (CCKBR): Rabbit anti—CCKBR polyclonal antibody, Antibodies-online, Aachen, Germany; catalog no. ABIN734318 (https://www.antibodies-online.com/antibody/734318/anti-Cholecystokinin+B+Receptor+CCKBR+AA+-301-400+antibody/). Source: KLH-conjugated synthetic peptide derived from human gastrin receptor. Purified by protein A affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Moller et al., 2017a).

4) Dopamine: Mouse anti-dopamine monoclonal antibody, Antibodies-online, Aachen, Germany; catalog no. ABIN721509 (https://www.antibodies-online.com/antibody/1721509/anti-Dopamine+DA+antibody/). Source: Derived from dopamine conjugated to bovine serum albumin. Purified by protein A affinity chromatography.

5) Dopamine transporter (DAT): Rabbit anti-DAT polyclonal antibody, Abcam, Cambridge, UK; catalog no. ab111468 (https://www.abcam.com/dopamine-transporter-antibody-ab111468.html). Source: KLH-conjugated synthetic peptide corresponding to mouse dopamine transporter aa 200–300. Immunogen affinity purification. Positive control: Brain (Kuang et al., 2021).

6) Dopamine receptor 2 (DRD2): Rabbit anti—DRD2 polyclonal antibody, Bioss, MA, USA; catalog no. bs-1008R (https://www.biossusa.com/products/bs-1008r). Source: KLH-conjugated synthetic peptide derived from human DRD2. Purified by Protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Moller et al., 2017a).

7) Dopamine receptor 5 (DRD5): Rabbit anti—DRD5 polyclonal antibody, Bioss, MA, USA; catalog no. bs-1747R (https://www.biossusa.com/products/bs-1747r). Source: KLH-conjugated synthetic peptide derived from human DRD5. Purified by Protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Moller et al., 2017a).

8) GABA: Mouse anti-GABA monoclonal antibody, Abcam, Cambridge, UK; catalog no. ab86186 (https://www.abcam.com/gaba-antibody-5a9-ab86186.html?productWallTab=Application&kapplications=74). Source: GABA coupled to BSA with glutaraldehyde. Purified by Protein G. Positive control: Brain (Mao and Pallas, 2013).  

9) GABA A receptor alpha (GABA_A_Ra): Rabbit anti—GABA_A_Ra polyclonal antibody, Abcam, Cambridge, UK; catalog no. ab33299 (https://www.abcam.com/gaba-a-receptor-alpha-1-antibody-a b33299.html). Source: Synthetic peptide corresponding to N terminal amino acids 1–15 of rat GABA_A_Ra. A cysteine at the C

![Schematic of the sensory neuroepithelium of the saccule.](https://www.antibodies-online.com/)
### Table 1

| Neurotransmitter / Receptor | General Location and Function | Findings in the Vestibular System (General) (Other Studies) | Findings in the Human Saccule (This Study) |
|----------------------------|--------------------------------|---------------------------------------------------------------|------------------------------------------|
| **Cholecystokinin** (CCK)  | CCK is found in both the central and peripheral nervous system, where it is involved in many basic functions such as satiety, nociception, regulation of body temperature, learning and memory as well as induction of fear and anxiety (Bohlen und Halbach and Dermietzel, 2006). CCK may be involved in brain disorders including Parkinson’s disease, Huntington’s chorea and schizophrenia. | Maller et al., 2017a demonstrated intense immunohistochemical reaction of CCKAR in the epithelial lining cells of the human endolymphatic sac (Maller et al., 2017a). Maller et al., 2017b found a moderate homogenous immunohistochemical reaction in the epithelial lining cells of the human endolymphatic sac | Intense expression of CCKAR in the non-sensory epithelial lining cells. No stromal expression found nor expression in the neuroepithelium. |
| **CCKAR**                  | CCKAR is a metabotropic receptor that elicits inhibitory postsynaptic responses but also short and prolonged excitation responses (Bohlen und Halbach and Dermietzel, 2006). | Maller et al., 2017b found a moderate homogenous immunohistochemical reaction in the epithelial lining cells of the human endolymphatic sac | Homogenous, granular CCKBR expression in the epithelial cells of the neuroepithelium, with intense peri-nuclear staining in some type 2-like hair cells. Sparse CCKBR expression in the stroma. No expression in the non-sensory epithelium. |
| **CCKBR**                  | CCKBRs are metabotropic receptors that preferentially elicit prolonged excitatory responses (Bohlen und Halbach and Dermietzel, 2006). | Maller et al., 2017b found a moderate homogenous immunohistochemical reaction in the epithelial lining cells of the human endolymphatic sac | Varying expression of dopamine in the subepithelial stroma, but no epithelial expression. |
| **Dopamine**               | The dopamine neurotransmitter and its receptors are widely distributed in the central nervous system, where they play a role in learning and memory, initiation and maintenance of motor function, goal-directed and reward-mediated behavior and regulation of vascular tone (Bohlen und Halbach and Dermietzel, 2006). They are involved in several brain disorders such as Parkinsonism, Alzheimer’s and schizophrenia (Bohlen und Halbach and Dermietzel, 2006; Brodal, 2016). Dopamine is synthesized in the presynaptic nerve terminals (Bohlen und Halbach and Dermietzel, 2006). | In the vestibular system, dopamine has shown to improve vestibular compensation, likely via a mechanism in the vestibular nuclei as described by Drago et al., 1996 (Drago et al., 1996). | Intense expression of the DAT in almost all epithelial lining cells. No stromal expression found, nor expression in the neuroepithelium. |
| **DAT**                    | The DAT is responsible for reuptake of dopamine (Bohlen und Halbach and Dermietzel, 2006). It is located not in the synaptic cleft but in perisynaptic areas why dopamine has to diffuse away before reuptake (Bohlen und Halbach and Dermietzel, 2006). | | |
| **DRD2**                   | | | There was no expression of the DRD2. |
| **DRD5**                   | | | There was no expression of the DRD5. |
| **GABA**                   | GABA is the major inhibitory neurotransmitter of the central nervous system, where it is widely distributed (Bohlen und Halbach and Dermietzel, 2006; Brodal, 2016). | GABA-like immunoreactivity has been confined to vestibular efferents and the synapse of the efferent nerves with the hair cells of human and rat otolithic organs and semicircular canals (Didier et al., 1990; Kong et al., 1998a, 1998b; Schrott-Fischer et al., 2002; Usami et al., 1987). Other authors have suggested that GABA may be involved in afferent vestibular signal transmission based on presence of GABA synthesizing enzymes in hair cells and GABA degradation enzymes in the afferent synapse and vestibular ganglion cells of chicken and guinea pig (López et al., 1992, 1995; Usami et al., 1989). It is established based on studies in a wide range of species that GABAergic inhibitory signaling plays a role in the peripheral vestibular system, and that GABAergic signaling from the cerebellum, inferior olivary nucleus and contralateral vestibular nuclei regulates vestibular nuclei processing (Foster et al., 1995; Kong et al., 1998a, 1998b; López et al., 1992; Schrott-Fischer et al., 2002; Shao et al., 2012; Usami et al., 1989). | | No expression of the GABA neurotransmitter was seen. |
| **GABA_A^a**               | GABA_A^a receptors are ligand-gated chloride channels that generate fast synaptic inhibition (Shao et al., 2012). The receptors are more commonly found in the forebrain than GABA_A receptors (Holstein, 2000). Both are present in a high concentration in the cerebellum (Holstein, 2000). | Punctate staining associated with the calyces and afferent nerves of crista ampullares (vestibular end organ of the semicircular canals) in rodents, have suggested that GABA_A may act to modify afferent nerve transmission via GABA released from e.g. hair cells, afferent or efferent nerves (Foster et al., 1995). GABA_A has also been demonstrated in the vestibular ganglion and in the vestibular nuclei of mammals (Foster et al., 1995; Shao et al., 2012). Behavioral studies have shown that GABA_A agonists and antagonists modify symptoms after vestibular | Expression of GABA_A^a along the neuroepithelial basal membrane, with projections into the neuroepithelial layer. Sparse expression was seen in the stroma, none in the non-sensory epithelial lining. |

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Table 1 (continued)

| Neurotransmitter / Receptor | General Location and Function | Findings in the Vestibular System in General (Other Studies) | Findings in the Human Saccule (This Study) |
|-----------------------------|-------------------------------|-------------------------------------------------------------|------------------------------------------|
| GABA<sub>B2</sub>R | GABA<sub>B</sub> receptors are G-protein-coupled receptors that produce slow, prolonged inhibition (Shao et al., 2012). In comparison with GABA<sub>A</sub> receptors, GABA<sub>B</sub> receptors tend to be more abundant in various nuclei (Holstein, 2000). Both are present in a high concentration in cerebellum (Holstein, 2000). | GABA<sub>B</sub> receptors have been demonstrated pre- and postsynaptically in the vestibular nuclei of non-human mammals and play a role in vestibular compensation (Holstein et al., 1992; Shao et al., 2012). For example, GABA<sub>B</sub>-mediated inhibition of the medial vestibular nucleus has been shown to modulate spontaneous nystagmus, the vestibular-ocular reflex and static vestibular function in rat and monkey (Cohen et al., 1987; Heskin-Sweezie et al., 2018; Holstein et al., 1992; Magnusson et al., 2002, 2003). | GABA<sub>B2</sub>R was expressed in the neuroepithelium in the transition zone towards the non-sensory epithelial lining. No expression in the stroma. |

L-glutamate | Glutamate is an excitatory neurotransmitter widely distributed in the central nervous system and in the ganglion cells of the peripheral nervous system (Bøhlen and Halbach and Dermietzel, 2006; Bødal, 2016). Glutamate potentiates fast synaptic transmission as well as long-lasting changes in neuronal excitability (Bøhlen and Halbach and Dermietzel, 2006; Bødal, 2016). It is responsible for cognition including learning and memory, motor functions and perception (Bødal, 2016). Excess glutamate is toxic to brain tissue due to hyperactivation and has been linked to disease processes such as ischemia, hypoglycemia, epileptic seizures and neurodegenerative diseases including Alzheimer’s disease and Parkinson (Bøhlen and Halbach and Dermietzel, 2006). | L-glutamate is found in the subepithelial stroma, corresponding to traversing nerve fibers. No epithelial expression was found. |

GluR1 | GluR1 generates fast synaptic excitation and is widely distributed in the brain (Bøhlen and Halbach and Dermietzel, 2006). | GluR1 was predominantly expressed in the subepithelial stroma of the saccule, but also in some cells of the neuroepithelium (type I-like hair cells), and in some cells of the non-sensory epithelial lining. |

mGluR | mGluR is a pre- and postsynaptic metabotropic receptor that produces long-lasting changes to neuronal excitability (Bøhlen and Halbach and Dermietzel, 2006; Bødal, 2016). It is present in the central nervous system, where it is thought to be involved in learning and memory (Bøhlen and Halbach and Dermietzel, 2006; Bødal, 2016). It is likely involved in brain diseases such as epilepsy, schizophrenia and stroke (Bødal, 2016). | The mGluR was expressed in type I-like hair cells of the neuroepithelium and in some epithelial lining cells. No stromal expression was found. |

H<sub>R</sub> | H<sub>R</sub> is a presynaptic receptor inhibiting release of acetylcholine, dopamine, GABA, glutamate, noradrenaline, and serotonin (Schlicker et al., 1994; Schlicker and Kaffmann, 2017). It is primarily located in the cerebral hemispheres of the central nervous system but also in the cerebellum, brainstem and spinal cord (Schlicker and Kaffmann, 2017). It is involved in numerous behavioral functions including learning and memory, sleep-wake regulation, hunger and food intake, and susceptibility to seizures (Schlicker and Kaffmann, 2017). In the autonomic nervous system, it regulates sensory nerve fibers and release of sympathetic neurotransmitters and -peptides in e.g. heart, vessel and airway (Panula et al., 2015; Schlicker et al., 1994). | H<sub>R</sub> expression has been seen in both the epithelial and in the subepithelial lining (Dugl et al., 2008; Takumida et al., 2016). H<sub>R</sub> has been demonstrated in the maculae of the saccule and utricle and the crista ampullares of the semicircular canals. More specifically, it has been demonstrated in type I hair cells, the calyx and dimorphic vestibular afferents and subepithelial cells (Tritto et al., 2009; Takumida et al., 2016). In the vestibular nuclei, H<sub>R</sub> is located presynaptically on GABA and glycine neurons, where H<sub>R</sub> receptor activation modulates GABA and glycine release during vestibular compensation following unilateral labyrinthectomy (Bergquist et al., 2000; Tighelet et al., 2014, [Tighelet et al., 2006], 2007; Zhou et al., 2013). | There was no expression of the H<sub>R</sub>. Intense expression of the H<sub>R</sub> was found in epithelial lining cells of the saccule. No stromal expression, nor expression in the neuroepithelium. |

Serotonin | Serotonin-producing neurons are located almost exclusively in the brain stem (Bødal, 2016). Centrally, serotonin is involved in the reward system and fear- and anxiety-motivated behavior, | The neurotransmitter serotonin was not expressed in the human saccule. Strong 5-HT<sub>3</sub>R expression was found in the subepithelial stroma. No expression was demonstrated in the neuroepithelium or in the non-sensory epithelial lining. |

5-HT<sub>3</sub>R | In humans, endolymphatic sac epithelium has shown HT<sub>3</sub>R expression (Moller et al., 2017a). In other mammals, immunoreactivity for HT<sub>3</sub>R has been (continued on next page)
terminus links to a carrier protein. Purified by Protein A. Positive control: Brain (Scott et al., 2020).

10) GABA B receptor 2 (GABABR2): Rabbit anti-GABABR2 polyclonal antibody, Abcam, Cambridge, UK; catalog no. ab52248 (https://www.abcam.com/gaba-b-receptor-gabbr2-antibody-ab52248.html?productWallTab=Abreviews). Source: Synthetic peptide corresponding to human GABABR2, purified by immunogen affinity chromatography. Positive control: Spinal cord and ganglia (Thibault et al., 2014).

11) L-Glutamate: Rabbit anti-L-glutamate polyclonal antibody, Abcam, Cambridge, UK; catalog no. ab9440 (https://www.abcam.com/l-glutamate-antibody-ab9440.html). Source: Small molecule corresponding to l-glutamate, purified by IgG fraction. Positive control: Brain, spinal cord, retina (Shivashankar et al., 2020).

12) Glutamate receptor 1 (GluR1): Rabbit anti-GluR1 monoclonal antibody, Abcam, Cambridge, UK; catalog no. ab109450 (https://www.abcam.com/glutamate-receptor-1-ampa-subtype-antibody-epxr15479-ab109450.html). Source: Synthetic peptide purified by Protein A. Positive control: Brain (Guo et al., 2020).

13) Metabotropic glutamate receptor (mGluR): Chicken anti-mGluR polyclonal antibody, Abcam, Cambridge, UK; catalog no. ab153669 (https://www.abcam.com/mglur1-antibody-ab153669.html?productWallTab=ShowAll). Source: KLH-conjugated synthetic peptide corresponding to human mGluR, purified by immunogen affinity. Positive control: Brain and ganglia (Abcam, Cambridge, UK, 2022).

14) Histamine receptor 1 (H3R): Rabbit anti-H3R polyclonal antibody, Bioss, MA, USA; catalog no. bs-6663R (https://www.biossusa.com/products/bs-6663r). Source: KLH-conjugated synthetic peptide derived from human H3R and purified by Protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Møller et al., 2016).

15) Histamine receptor 3 (H4R): Rabbit anti-H4R polyclonal antibody, Bioss, MA, USA; catalog no. bs-3635R (https://www.biossusa.com/products/bs-3635r). Source: KLH-conjugated synthetic peptide derived from human H4R and purified by Protein A and peptide affinity chromatography. Positive control, previous study by our group: Endolymphatic sac (Møller et al., 2016).

16) Serotonin: Mouse anti-serotonin monoclonal antibody, Antibodies-online, Aachen, Germany; catalog no. ABIN2358246 (https://www.antibodies-online.com/antibody/2358246/antibody/). Source: Derived from cytoplasmic 5-hydroxytryptamine hydrochloride purified using supernatant.

17) Serotonin receptor 1D (5-HT1D): Rabbit anti-5-HT1D polyclonal antibody, Antibodies-online, Aachen, Germany; catalog no. ABIN122599. Source: KLH-conjugated synthetic peptide, purified by peptide immunoaffinity. Positive control, previous study by our group: Endolymphatic sac (Møller et al., 2017a).

For all antibodies, dilutions 1:50, 1:100, 1:200, 1:300, 1:500, 1:1000 were used. The dilutions 1:300 and 1:500 were found to be adequate for all antibodies.

2.5. Normal temporal bone

For orientation and illustration, a HE stained, cellloidin embedded section from a normal decalcified human temporal bone showing the vestibule/saccule was included. This postmortem sample was randomly chosen among normal temporal bones in the archives of the departmental temporal bone collection.

3. Results

3.1. Histological morphology of the human saccule

Fig. 3A provides an overview of the middle and inner ear, including the vestibule, the saccule and the sacular macula of the vestibular system from a normal human temporal bone. Details of the sub-epithelial stroma of the macula, the neuro-epithelium and the kinocilia/gelatinous mass layer are shown (Fig. 3C), as is the vestibular ganglion (Fig. 3B). Inevitably, autolysis occurs before a temporal bone can be removed and fixed post-mortem, leading to blurring of histomorphological details, as seen in Fig. 3C.

The freshly sampled and fixed human sacculus provided a morphological reference for the tissue sections labelled by immunohistochemistry, as well as non-autolyzed details of histomorphology (Figs. 4A & 5A). The sacular maculae were readily displayed with minimal artifacts, showing the pseudostratified, ciliated neuroepithelium and sub-epithelial stroma, containing the afferent and efferent nerve fibers. The single-layered non-sensory epithelium of the saccule was supported by only a thin subepithelial stroma and thus subject to collapse and extensive folding during harvesting and paraffin embedding (Fig. 5A). The transition zone between the neuroepithelium and lining epithelium is also displayed in Fig. 5A.

3.2. Immunohistochemical findings, overview

Immunohistochemistry demonstrated expression of eleven of the

| Table 1 (continued) |
|---------------------|
| About the neurotransmitter / receptor: general location and function | Findings in the vestibular system in general (other studies) | Findings in the human saccule (this study) |
|---------------------|
| whereas it peripherally produces smooth muscle concentration, elevates the concentration of platelets, etc. (Ibrudal, 2016; Kranz et al., 2011; Mykter, 2003). From studies in mammals including humans, 5-HT1D-R has been shown to function as an autoreceptor that regulates release of the serotonin neurotransmitter (Bohlen and Halbach and Dermietzel, 2006). Triptans used for treating migraine, are 5-HT1D-R agonists (Ahn and Balaban, 2010; Bohlen and Halbach and Dermietzel, 2006). 5-HT1D-R is also thought to be involved in neuronal diseases such as Alzheimer’s disease and Huntington’s-Chorea (Bohlen and Halbach and Dermietzel, 2006; García-Alcolea et al., 2004). | demonstrated in vestibular ganglion cells (Ahn and Balaban, 2010). |
seventeen selected neurotransmitters and neurotransmitter receptors in the human saccule (Tables 1 & 2). No staining was seen in the negative controls.

The saccular neuroepithelium expressed GluR1, mGluR, CCKBR, GABA\textsubscript{A}R\textsubscript{x} and GABA\textsubscript{B}R\textsubscript{2} (Fig. 4B-F), whereas the non-sensory epithelial lining expressed primarily CCKAR, DAT and H\textsubscript{3}R (Fig. 5B-D), but sporadically also GluR1 and mGluR (not shown). The subepithelial stroma expressed primarily GluR1 and 5-HT\textsubscript{1D}R (Fig. 4E & G), to a lesser extent also GABA\textsubscript{A}R\textsubscript{x} and CCKBR (Fig. 4B & F), as well as L-glutamate and dopamine (not shown).

We found no expression of cholecystokinin, serotonin and GABA, nor of receptors DR2, DRD5 and H\textsubscript{1}R.

3.3. Expression of GABA, GABA\textsubscript{A}R\textsubscript{x} and GABA\textsubscript{B}R\textsubscript{2}

As noted, both GABA receptors were expressed in the saccule. More specifically, GABA\textsubscript{A}R\textsubscript{x} was expressed sporadically in the stroma, but most intensely along the basement membrane of the neuroepithelium, with projections into the epithelium (Fig. 4B). GABA\textsubscript{B}R\textsubscript{2} was expressed in the transition zone between the neuroepithelium and non-sensory epithelium (Fig. 4C). We found no expression of the GABA neurotransmitter.

3.4. Expression of cholecystokinin, CCKAR and CCKBR

Homogenous, granular expression of CCKBR was seen within the neuroepithelial cells, with intense perinuclear labelling in what morphologically resembles type 2-like hair cells (Fig. 4F). There was also sparse, sporadic expression in the subepithelial stroma. CCKAR was expressed in the non-sensory epithelium (Fig. 5B). No expression of the cholecystokinin neurotransmitter was seen.

3.5. Expression of glutamate, mGluR and GluR1

L-glutamate was expressed only in the subepithelial stroma, where the most prominent GluR1-labelling was also seen. Some cells of the neuroepithelium, morphologically corresponding to type 1 hair cells, and some cells of the non-sensory epithelial lining also expressed GluR1 (Fig. 4E). mGluR was located in the neuroepithelium, morphologically corresponding to type 1 hair cells (Fig. 4D), and in the non-sensory epithelium with varying intensity. No expression was found in the stroma.

3.6. Findings in the non-sensory epithelium and subepithelial stroma

In addition to CCKAR (Fig. 5B), the non-sensory epithelial lining demonstrated intense expression of H\textsubscript{3}R and varying expression of DAT (Fig. 5C & D). As mentioned above, some of these cells also expressed GluR1 and mGluR.

Abundant expression of 5-HT\textsubscript{1D}R was demonstrated in the subepithelial stroma (Fig. 4G), whereas no expression of the serotonin neurotransmitter was demonstrated. Finally, we found varying expression of dopamine, in the subepithelial stroma only.

4. Discussion

4.1. Summary of findings

This study describes neurotransmitter and neurotransmitter receptor expression in freshly harvested saccules from the human vestibular system. We demonstrate expression of GluR1, mGluR, CCKBR, GABA\textsubscript{A}R\textsubscript{x} and GABA\textsubscript{B}R\textsubscript{2} in the saccular neuroepithelium, and of GluR1, mGluR, CCKAR, DAT and H\textsubscript{3}R in the non-sensory epithelial lining of the saccule. In the subepithelial stroma, through which the neuronal afferents and efferents of the saccular neuroepithelium traverse, we demonstrate expression of L-glutamate, GluR1, CCKBR, GABA\textsubscript{A}R\textsubscript{x}, 5-HT\textsubscript{1D}R and dopamine. We do not find expression of the cholecystokinin, serotonin and GABA neurotransmitters, or of the receptors DR2, DRD5, H\textsubscript{1}R (Table 1 & 2).

4.2. Other studies on neurotransmitters in the human vestibular system

Very few studies have investigated neurotransmitter and neurotransmitter receptor expression in the peripheral vestibular system of humans. In the vestibular sensory organs, only GABA expression has been studied (Kong et al., 1998a, 1998b; Schrott-Fischer et al., 2002), whereas neurotransmission related to the endolymphatic sac has been explored somewhat more extensively (Møller et al., 2016, 2017a).

4.3. Contribution to the understanding of neurotransmission in the peripheral vestibular system

Glutamate is thought to be the major excitatory neurotransmitter of the vestibular system. Animal studies have demonstrated glutamate-like reactivity in the vestibular hair cell synapse, both pre- and
postsynaptically, in nerve fibers and in the vestibular ganglion cells (Dememes et al., 1990; Guth et al., 1998b; Harper et al., 1995; Soto and Vega, 1988). Fast afferent signal transmission likely involves GluR1, an ionotropic receptor, which has been identified postsynaptically in the type I hair cell-calyx complex in rat and guinea pig (Bohlen und et al., 2006; Dememes et al., 1995). The mGluR, a metabotropic receptor that produces long-lasting changes in excitability, has been demonstrated presynaptically in the afferent hair cell synapse (Bohlen und et al., 2006; Guth et al., 1998a). mGluR has also been found in vestibular ganglion cells, where it likely plays a role in modulating the afferent signal (Andrianov et al., 2005; Kong et al., 1998a). In the human saccular tissue, we demonstrated GluR1 in the neuroepithelium, morphologically corresponding to type I-like hair cells, consistent with the receptor being involved in afferent and/or efferent transmission. The expression was far more abundant in the subepithelial stroma (through which afferent and efferent nerve fibers traverse), which indicates predominantly afferent transmission. We also found expression of mGluR in type I-like hair cells of the neuroepithelium, but none in the subepithelial stroma, indicating a role in efferent signal transduction or in presynaptic regulation of the afferent hair cell synapse as demonstrated by other studies. Labeling for L-glutamate was only seen in the subepithelial stroma, also consistent with a role in efferent signaling. Thus, our findings suggest that glutamate is involved in both afferent and efferent signal transduction in the human saccule.

The GABA neurotransmitter and its inhibitory receptors are thought to play an important role in processing of vestibular signals: first of all via efferent signaling that modulates the afferent response and secondly, in integration of input from the ipsilateral and contralateral vestibular organs, cerebellum, etc. in the vestibular nuclei (Foster et al., 1995; Kong et al., 1998a, 1998b; López et al., 1992; Schrott-Fischer et al., 2002; Shao et al., 2012; Usami et al., 1989). Previous studies on human and non-human mammal otolithic and semicircular canal organs have demonstrated GABA-like immunoreactivity in what are presumably vestibular efferents synapsing with the type I hair cell calyx (Kong et al., 1998a, 1998b; Schrott-Fischer et al., 2002; Usami et al., 1987). These studies did not find GABA-like immunoreactivity in hair cells, calyceal nerve terminals or afferent fibers and suggested that GABA is responsible for efferent signaling, modifying the afferent response (Kong et al., 1998a, 1998b; Schrott-Fischer et al., 2002). In this study, we did not demonstrate presence of the GABA neurotransmitter in the human...
saccule. However, GABA_B receptor 5, GABA, gamma-aminobutyric acid; GABA_A receptor B; DAT, dopamine transporter; DRD2, dopamine receptor 2; DRD5, dopamine receptor 5, presence of neurotransmitter or neurotransmitter receptor; ( ), varying expression; CCKAR, cholecystokinin receptor A; CCKBR, cholecystokinin receptor B; DAT, dopamine transporter; DRD2, dopamine receptor 2; DRD5, dopamine receptor 5, GABA, gamma-aminobutyric acid; GABA_A receptor alpha; GABA_B receptor 2; GluR1, glutamate receptor 1; mGluR, metabotropic glutamate receptor; H_3R, histamine receptor 3; H_3R modulate afferent and/or efferent signals to and from the sensory cells in the vestibular end organs. Future studies may elucidate whether triptans have a site of action in the vestibular periphery, in addition to sites of action demonstrated in the vestibular ganglion, vestibular nuclei and other central locations (Ahn and Balaban, 2010; Balaban, 2016; Balaban et al., 2011; Tepper et al., 2002).

Of special interest, this study found intense expression of the histamine receptor H_3R in the non-sensory epithelial lining cells of the saccule, whereas there was no expression in the neuroepithelium, nor in the subepithelial stroma. The H_3R has previously been demonstrated in the otolithic and semicircular canal neuroepithelium in non-human mammals, where it was primarily located in type I hair cells and subepithelial cells (Takumida et al., 2016). It has also been identified in the vestibular afferents, and in the epithelium and subepithelial capillary network of the endolymphatic sac in mammals, including humans (with some contradictory findings in the endolymphatic sac epithelium) (Dagli et al., 2008; Moller et al., 2016; Takumida et al., 2016; Tritto et al., 2009). The H_3R receptor is a presynaptic inhibitory metabotropic receptor (Bohlen und et al., 2006). It is primarily located in the central nervous system, where it is involved in numerous behavioral functions such as learning, memory, sleep-awake regulation and hunger, as well as regulation of hair cells in the central versus peripheral macular regions. A study on rodent crista ampullares of the semicircular canals showed punctate staining of GABA_B receptor 3; 5-HT_1D, 5-HT receptor 1D.

The expression pattern of the two receptors also suggests differential regulation of hair cells in the central versus peripheral macular regions. In migraine, including vestibular migraine, triptans are used to attenuate symptoms during an acute attack (Balaban et al., 2011). Triptans have different targets including agonist action on the 5-HT_1D, a metabotropic receptor that regulates neurotransmitter and -peptide release of e.g. dopamine (Ahn and Balaban, 2010; Bohlen und et al., 2006). We found strong expression of the 5-HT_1D in the stroma basal to the saccular neuroepithelium. It is possible that serotonin and the 5-HT_1D modulate afferent and/or efferent signals to and from the sensory cells in the vestibular end organs. Future studies may elucidate whether triptans have a site of action in the vestibular periphery, in addition to sites of action demonstrated in the vestibular ganglion, vestibular nuclei and other central locations (Ahn and Balaban, 2010; Balaban, 2016; Balaban et al., 2011; Tepper et al., 2002).

Table 2
Expression of neurotransmitters and neurotransmitter receptors in the human saccule.

| The Saccule          |
|----------------------|
|                     |
| Cholecystokinin      |
| CCKAR                |
| CCKBR                |
| Dopamine             |
| DAT                  |
| DRD2                 |
| DRD5                 |
| GABA                 |
| GABA_A receptor alpha|
| GABA_B receptor 2    |
| L-glutamate          |
| GluR1                |
| mGluR                |
| H_3R                 |
| H_3R                 |
| Serotonin            |
| 5-HT_1D              |
| +                    |
| +                    |
| ( )                 |
| ( )                 |
| ( )                 |
| +                    |
| +                    |
| ( )                 |
| ( )                 |
| +                    |

(+), presence of neurotransmitter or neurotransmitter receptor; ( ), varying expression; CCKAR, cholecystokinin receptor A; CCKBR, cholecystokinin receptor B; DAT, dopamine transporter; DRD2, dopamine receptor 2; DRD5, dopamine receptor 5, GABA, gamma-aminobutyric acid; GABA_A receptor alpha; GABA_B receptor 2; GluR1, glutamate receptor 1; mGluR, metabotropic glutamate receptor; H_3R, histamine receptor 3; 5-HT_1D, serotonin receptor 1D.

saccule. However, GABA_A receptor alpha (Bohlen und et al., 2006), was found along the basement membrane of the neuroepithelium with projections into the epithelium. GABA_B receptor 2, a metabotropic receptor (Bohlen und et al., 2006), was also expressed in the neuroepithelium, in what is likely the transmission zone. The expression of these receptors could reflect efferent transmission, as suggested by the previous studies.
in the autonomic nervous system, where it has a regulatory role related to the heart, vessels and airways (Schlicker et al., 1994; Schlicker and Kathmann, 2017). This study could not confirm the presence of H3R in the neuroepithelium, and thereby a role in regulating afferent signaling. However, the receptor was expressed intensely in the non-sensory epithelium. This could indicate a role in fluid homeostasis, as has been hypothesized for the endolymphatic sac (Moller et al., 2016). In a pharmacological perspective, these saccular epithelial lining receptors may be a target in treatment with betahistine, a H3R antagonist used for Menière’s disease (Gbabou et al., 2010). The H3R receptor, and thereby betahistine targets, have already been documented in many other locations along the vestibu-lo-cochlear pathway, e.g. in vestibular ganglion cells and vestibular nuclei, and in the stria vascularis and endolymphatic sac (Bergquist et al., 2006; Dagli et al., 2008; Ihler et al., 2012; Taku mida et al., 2016; Tighilet et al., 2007; Tighilet and Lacour, 1996; Zhou et al., 2013).

In addition to H3R, we also found that the non-sensory saccular epithelium expressed CCKAR. Previous findings suggest that CCK can regulate ion transport; more specifically, a study in rabbit kidney showed that CCK increased sodium excretion and decreased excretion of magnesium and calcium (Duggan et al., 1988). It has been hypothesized that CCK release from the endolymphatic sac in response to fluid changes may act to maintain the pivotal fluid homeostasis in the inner ear via a paracrine mechanism on CCKAR and CCKBR within the endolymphatic sac epithelium, as well as in other areas of the labyrinth (Moller et al., 2017a). Activation of CCKAR and CCKBR is thought to alter transepithelial ion and fluid transport, thus reestablishing fluid homeostasis (Moller et al., 2017a). We found a homogenous, granular expression of CCKBR in the saccular neuroepithelium, with intense peri-nuclear labelling in some type II-like hair cells, which indicates that this receptor is involved in saccular neurotransmission. Both CCKAR and CCKBR are metabotropic receptors with both excitatory and inhibitory effects, and they are found in both the central and peripheral nervous system (Bohlen und et al., 2006). They are involved in many basic functions such as satiety, nociception, regulation of body temperature, learning and memory as well as induction of fear and anxiety (Bohlen und et al., 2006). Their neurotransmitter CCK has also been found in sensory cells of the non-human mammalian saccule (Nowak et al., 1986).

The dopamine neurotransmitter and its receptors are widely distributed in the central nervous system, where they are involved in learning and memory, initiation and maintenance of motor function, goal-directed and reward-mediated behavior and regulation of vascular tone (Bohlen und et al., 2006). The dopamine system is involved in several brain disorders including Parkinsonism, Alzheimer’s and schizophrenia (Bohlen und et al., 2006). In the vestibular system, dopamine has shown to improve vestibular compensation, likely via a mechanism in the vestibular nuclei (Drago et al., 1996). In the cochlea, dopamine plays an important role as an efferent transmitter that suppresses afferent activity thereby controlling noise-induced excitotoxicity (Maison et al., 2012; Ruel et al., 2006, 2001). Dopamine may play a similar protective role in the peripheral vestibular system, including the saccular macula, in which we demonstrated expression in the subepithelial stroma. DAT was expressed in the non-sensory lining epithelium, which indicates that it may be involved in ion/fluid transport, thus potentially playing a role in inner ear homeostasis, as for H3R and CCKAR.

4.4. Perspectives on pharmacology

The present findings provide information for interpretation of both effects and side-effects of medical treatment with drugs that bind to or affect the functionality of the neurotransmitter receptors explored, e.g. treatment with peroral histamine. Findings do also provide perspectives for future potential drug targets aimed at modulation of vestibular function, e.g. inhibition in case of vertigo or stimulation when aiming at improving vestibular function after various diseases, e.g. vestibular neuritis or Meniere’s disease. In that context, local administration of drugs into the middle ear is an interesting and increasingly used alternative to systemic administration, as this leads to higher concentration drug within the inner ear and less side effects in general (Salt and Plontke, 2009).

4.5. Potential biases

We sampled the saccule freshly from the vestibule of patients with a vestibular schwannoma. No patients had tumor components within the labyrinth of the temporal bone, as all tumors resided in the cerebello-pontine angle, with a varying component extending into the inner ear canal. Thus, we estimate that the likelihood of direct tumor influence on saccular expression of transmitters / receptors is negligible, but it cannot be entirely ruled out that the tumor may have interfered with the function of nerve fibers and thus the expression of transmitters and receptors in the saccule.

Since this study did not use markers for specific cell types, expression of neurotransmitters and receptors in specific hair cells or synapses are only estimates based on morphology and expression pattern.

5. Conclusion

Applying immunohistochemistry to freshly harvested tissue, this study is the first to demonstrate expression of several neurotransmitters and neurotransmitter receptors in the saccule of the human vestibular system. The neuroepithelium expressed GluR1, mGluR, GABAαR, GABAγR2 and CCKBR. The subepithelial stroma, through which afferent and efferent saccular nerve fibers traverse, expressed 5-glutamate, GluR1, CCKBR, GABAAR, dopamine and 5-HT3R. Finally, GluR1, mGluR, H3R, CCKAR and DAT were expressed in the non-sensory lining epithelium. There was no expression of the neurotransmitters: GABA, cholecystokinin and serotonin, or of the receptors: DR2, DRD5 and H1R.

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Data statement

Data available upon request

Author contributions

KEE: Analysis, Writing – Original Draft, Writing – Review & Editing, Visualization.
SK: Investigation (performed the immunohistochemical preparation and staining), Writing – Review & Editing.
LJH: Investigation (normal temporal bone), Writing – Review & Editing.
PCT: Conceptualization, Investigation (sampled saccular tissue), Analysis, Writing – Review & Editing, Visualization, Supervision.

All authors approved the final and submitted version of the manuscript.

Declaration of Competing Interest

None.

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Appendix A: The peer review overview and supplementary data

The Peer Review Overview and Supplementary data associated with this article can be found in the online version, at: doi: 10.1016/j.pneurobio.2022.102238

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