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CELLULAR CORRELATES OF SENSORY PROCESSING IN THE MAMMALIAN AUDIO-VESTIBULAR BRAINSTEM

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Cellular Correlates of Sensory Processing in the Mammalian Audio-Vestibular Brainstem

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Try to learn something about everything, and everything about something."
Thomas Huxley

I am naturally curious and I love to learn. I love traveling to distant places I have never seen, experiencing new cultures, and communicating in new languages. I guess that's my 'something about everything'. I also have an inborn curiosity about the world and how it functions. I guess that is why science has become my 'everything about something'.

Dedicated to my mother.

For always encouraging me to stay curious; no matter where or how far that curiosity will lead me.

ABSTRACT

Efferent projections to the vestibular inner ear organs have remained elusive. To shed light on their physiological role, a first investigation of the vestibular efferent (VE) neurons in the brainstem was undertaken by using a transgenic mouse which expresses a fluorescent marker in the VE neurons. The intrinsic electrical properties of VE neurons were compared to those of the lateral olivocochlear (LOC) brainstem neurons, which innervate the cochlea. The study demonstrated that, due to more complex expression of potassium-based conductances, VE neurons display a more bimodal firing pattern than LOC neurons, which indicates that their role may be more widespread and control both motion and gravity sensors (Paper I). This thesis next investigated the cellular properties of the superior paraolivary nucleus (SPON) neurons in normal (Paper II and III) and congenitally deaf (Paper IV) mice. This evolutionary conserved mammalian brainstem structure has been implicated in the processing of speech cues by extracting the temporal signal in coarse sound amplitude fluctuations or brief sound segments, by responding abruptly to the offset of a tone stimulus or by entrainment to slow amplitude modulations of the same tone. Patch-clamp recordings in brain slices revealed that all SPON neurons exhibit postinhibitory rebound spiking, generated by the subthreshold-activated h current and low voltage-activated calcium current of the T-type. Pharmacological blockade of these currents in vivo abolished the sound-induced offset response and sensitivity to amplitude modulated tones, providing evidence that rebound spiking is the mechanism for offset-spiking in SPON (Paper II). In addition to a powerful inhibitory input, SPON was also confirmed to receive a single excitatory input from the octopus cells (Paper III) – held to be the most temporally precise neurons in the brain, responding with extremely high precision to complex sounds. A selective, strong projection from the octopus cells can also explain why SPON responds to the onset of sounds and is compatible with the idea that there are specialized brain circuits that encode the slow temporal rhythm contained in natural sounds, such as speech. The robustness of these brain circuits was demonstrated in SPON of congenitally deaf mice. Despite the absence of input activity, the deaf SPON neurons developed normal capacity for well-timed rebound spiking. This remarkable rescue of the SPON cellular function may have been possible due to up-regulation of the neuroprotective factor neuritin, prolonging the developmental time window (Paper IV).

In summary, this thesis demonstrates, on a cellular level, how combinations of different voltage-gated ion channels that are activated by excitation or inhibition or both, can create distinct firing patterns in sensory neurons that encode selective features of the incoming afferent signal. This code will either project back to control the sensory receptors or feed into higher order brain areas where it contributes to the hierarchical processing that enable us to perceive and comprehend a sensation.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. **Leijon S** and Magnusson AK (2014) Physiological Characterization of Vestibular Efferent Brainstem Neurons Using a Transgenic Mouse Model. *PLoS One*. 9, e98277

II. Felix II RA, Fridberger A, **Leijon S**, Berrebi AS and Magnusson AK (2011) Sound rhythms are encoded by postinhibitory rebound spiking in the superior paraolivary nucleus. *Journal of Neuroscience*. 31, p 12566-78.

III. Felix II RA, Gourévitch B, **Leijon S**, Magnusson AK (2015) Octopus cells in the posteroventral cochlear nucleus provide a robust excitatory input to the superior paraolivary nucleus. *Manuscript.*

IV. **Leijon S**, Peyda S and Magnusson AK (2015) Temporal processing capacity in auditory-deprived superior paraolivary neurons is rescued by sequential plasticity during early development. *Submitted.*

CONTENTS

LIST OF ABBREVIATIONS

ABR   Auditory brainstem response

AC   Auditory cortex

aCSF   Artificial cerebrospinal fluid

AVCN   Anteroventral cochlear nucleus

ChAT   Choline acetyltransferase

CI   Cochlear implant

CN   Cochlear nucleus

CNS   Central nervous system

DCN   Dorsal cochlear nucleus

eGFP   Enhanced green fluorescent protein

EPSP/C   Excitatory postsynaptic potential/current

EVS   Efferent vestibular system

HRP   Horseradish peroxidase

IAS   Intermediate acoustic stria

IC   Inferior colliculus

LOC   Lateral olivocochlear

LSO   Lateral superior olive

MGB   Medial geniculate body

MOC   Medial olivocochlear

MNTB   Medial nucleus of the trapezoid body

MSO   Medial superior olive

NIII   8th cranial nerve

NLL   Nucleus of the lateral lemniscus

SOC   Superior olivary complex

PBS   Phosphate buffered saline

PCR   Polymerase chain reaction

PNS   Peripheral nervous system

PVCN   Posteroventral cochlear nucleus

SPON   Superior paraolivary nucleus

VCN   Ventral cochlear nucleus

VE   Vestibular efferent

VNTB   Ventral nucleus of the trapezoid body

1 INTRODUCTION

Although our hearing and balance comprise two independent sensory systems, both systems work on the principle of conveying sensory information from peripheral inner ear end organs to the brain via temporally precise action potentials. Projection fibers transmitting action potentials from balance (vestibular) and hearing (auditory) end organs make up the 8th cranial nerve (NVIII), which enters the central nervous system at the level of the brainstem. Here the projection pathways physically diverge, as vestibular and auditory fibers contact separate brainstem nuclei. For the central neurons to make use of the vast information contained in the sensory signals, they are specialized to extract and process distinct features regarding gravity and sound waves. Apart from differences in projection patterns, which directly affects the information that reaches a receiving neuron, another specialization is to modify neuronal function on a cellular level by differential expression of specific ion channels. Subtle differences in the combination of ion channels enables a neuron to more precisely fine-tune how the received signal is finally transmitted as an output function.

As our hearing and balance are both evolutionarily old, this has over time allowed them to evolve into highly complex systems. Although the beauty and complexity of the sensory end organs has long been admired and investigated by curious physicians and scientists alike, the exact mechanisms of auditory and vestibular central processing still remain poorly understood. Utilizing a combination of immunohistochemical and electrophysiological techniques I have investigated cellular mechanisms that provide the basis for vestibular and auditory function. More specifically, I herein present novel findings that contribute to our understanding of mammalian vestibular sensory feedback to the inner ear, as well as mammalian encoding of communication sounds in normal and deaf individuals.

1.1 AUDIO-VESTIBULAR EFFERENCE

Neurons that receive information from our sensory end organs and convey these signals to the central nervous system (CNS) are called afferent neurons. The projection fibers of the afferent neurons make up the ascending CNS pathways, essential for turning a peripheral stimulus into a sensory experience. However, the CNS also sends feedback to the periphery via efferent neurons in descending pathways. This audio-vestibular efference exerts control over the cochlea and the vestibular apparatus and, thus, provides the CNS with a mechanism of modulating afferent input in response to sensory stimuli.
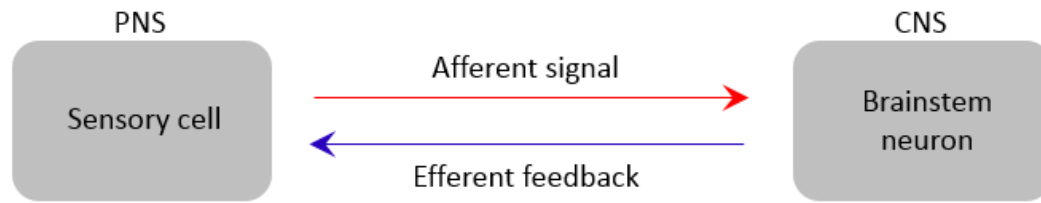
Figure 1.1. Simplified schematic basis for efferent control. Sensory stimuli evoked in sensory cells in the peripheral nervous system (PNS) are transmitted to the brain via afferent neurons. Efferent neurons in the central nervous system (CNS) project back to the sensory epithelium and contribute to shaping afferent responses.

1.1.1 Vestibular efference – VE

The existence of an efferent vestibular system (EVS) was demonstrated over fifty years ago by Rasmussen and Gacek (1958). However, the origin of that efferent innervation for long remained a mystery. It was not until Gacek and Lyon (1974) and Warr (1975) in two separate studies performed intra-labyrinthie injections with the retrograde tracer horseradish peroxidase (HRP) in newborn kittens, that the anatomical location of the cells providing the efferent innervation to the inner ear was elucidated. The vestibular efferent neurons have since been found in all studied vertebrates, including frogs (Birinyi et al., 2001), fish (Roberts and Meredith, 1992), birds (Whitehead and Morest, 1981; Code and Carr, 1994) and monkeys (Goldberg and Fernández, 1980; Carpenter et al., 1987). In rodents (White and Warr, 1983; Perachio and Kevetter, 1989; Shumilina and Preobrazhenskiĭ, 1990; Purcell and Perachio, 1997; Metts et al., 2006), vestibular efferent (VE) neurons comprise a small cholinergic group located dorsolateral to the genu of the facial nerve (NVII). They are small and few in number, with neuronal counts yielding total numbers <200.

Vestibular efferents send their axonal fibers laterally and as they leave the brainstem they converge with the olivocochlear efferent fibers and continue via the vestibulocochlear nerve (NVIII) to the inner ear (Goldberg and Fernández, 1980). Innervation of the vestibular neuroepithelium has been studied in detail by numerous investigators. Although VE neurons are few in number, they show rich arborization and make synaptic contact with all five vestibular end organs as first shown in rat (Kong et al., 1994). The efferent synaptic contacts have also been studied in human (Ishiyama et al., 1994; Kong et al., 1998) and with electron microscopy in chinchilla (Lysakowski and Goldberg, 1997), turtle (Holt et al., 2006), pigeon (Li et al., 2007) and squirrel monkey (Lysakowsi and Goldberg, 2008). Based on these studies VE neurons are confirmed to densely innervate the vestibular hair cells via three kinds of terminals (reviewed in Jordan et al., 2013). Efferent vesiculated bouton terminals synapse on type II vestibular hair cells and on bouton afferents innervating type II hair cells. As type I

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hair cells are engulfed by afferent calyces, they almost exclusively receive indirect efferent innervation, where efferent bouton terminals synapse on the afferent calyces. Furthermore, one efferent neuron can simultaneously provide innervation to type I and type II hair cells.

No previous studies have directly investigated the physiological properties of VE neurons, but several studies have recorded afferent responses to efferent stimulation (Goldberg and Fernández, 1980; Bernard et al., 1985; Boyle and Highstein, 1990; Highstein, 1991; Brichta and Goldberg, 2000; Marlinski et al., 2004; Holt et al., 2006; Boyle et al., 2009). Although differences in the direction of afferent responses to efferent stimulation is observed across vertebrate species, the effect is invariably excitatory in mammals (Jordan et al., 2013). Efferent activity gives a net increase in afferent discharge rate, resulting in a reduced gain. Interestingly, efferent activity commonly results in two separate excitatory components, namely a fast perstimulus excitation and a second much slower excitation that outlives the stimulus by tens of seconds (Goldberg and Fernández, 1980; Marlinski et al., 2004).

The functional role of the EVS was originally hypothesized as a means of quenching and modulating afferent responses arising from self-generated motions. This was based on studies in awake primates, displaying decreased sensitivity in central second order neurons of the vestibular nuclei during active (volitional) head movements (McCrea et al., 1999; Roy and Cullen, 2001). Such role would also be compatible with earlier results from toadfish, where VE neuron activity was associated with arousal and predation (Highstein, 1992). However, a seminal study in awake primates contradicted this idea as the afferent vestibular activity did not differ between actively (voluntary) or passively applied movements (Cullen and Minor, 2002). As a corollary, the functional role of the EVS remains elusive. Important clues for elucidating its functional role might however arise when studying the cellular properties of the VE neurons.

1.1.2 Auditory efference – LOC

As no *in vitro* recordings had previously been performed on VE neurons, their intrinsic properties and firing patterns were completely unknown. However, the efferent neurons projecting to the hearing end organ in the cochlea, the olivocochlear neurons, have been more extensively studied, and their functional roles are better understood. Since the vestibular and auditory sensory systems share a common phylogenetic origin, comparisons between the two efferent systems could prove useful for an advancement in our understanding of the functional role of the EVS.

There are two kinds of olivocochlear efferents, the medial olivocochlear efferents (MOC) and the lateral olivocochlear efferents (LOC). As for vestibular efferents, the

use of HRP retrograde labeling contributed greatly to the mapping of their localizations and projections. Both the MOC and LOC neurons reside in the ventral auditory brainstem, in the superior olivary complex (SOC). In rodents, the large multipolar MOC neurons reside within the ventral nucleus of the trapezoid body (VNTB) and the smaller fusiform LOC neurons in the lateral superior olive (LSO; White and Warr, 1983; Campbell and Henson, 1988). Similarly to the VE neurons, they both project via the vestibulocochlear nerve (NVIII) to the inner ear. The efferent innervation of cochlear hair cells has been more extensively studied in cat and guinea pig (Warr and Guinan, 1979; Liberman, 1980; Ginzberg and Morest, 1984; Robertson and Gummer, 1985; Liberman and Brown, 1986; Brown, 1987), than in rodents (Brown et al., 1991; Wilson et al., 1991). Thick MOC fibers cross the tunnel of Corti and terminate directly onto the outer hair cells in the cochlea, where they form large synapses. Thin unmyelinated LOC fibers enter the inner spiral or tunnel spiral bundle and terminate under the inner hair cells, where they synapse onto dendrites of afferent fibers. Occasionally, LOC fibers also synapse directly onto inner hair cells. While the density of MOC terminals peak around the middle of the cochlea, the LOC terminals are relatively evenly distributed along the cochlear axis.

The medial and lateral olivocochlear systems have different functional roles. Medial and lateral olivocochlear fibers intermingle with each other and with VE fibers at the level of the facial genu. However, as MOC fibers are the only ones to cross the midline, they, but not VE or LOC, can be selectively targeted by manipulations directly below the fourth ventricle. In combination with myelinated MOC fibers being thicker, thus more easily targeted, this has contributed to MOC becoming more commonly investigated. Innervating the outer hair cells, which function as biological amplifiers, MOC activity can modulate cochlear mechanics and basilar membrane motion, thereby playing a role in cochlear protection against loud sounds, detection and discrimination of sounds in noise and localization of sounds (reviewed in: Cooper and Guinan, 2006; Guinan, 2010; Rabbitt and Brownell, 2011). The LOC system is, similarly to the EVS, exclusively ipsilateral (Maison et al, 2003; Warr et al., 1997) and innervates the primary sensory hair cells. Furthermore, as for VE neurons, the direction of the effect that LOC activity has on afferent activity differs across species, as indicated by lesion studies (Le Prell et al., 2003; 2005; 2014; Darrow et al., 2007). The study by Darrow and colleagues indicates that the net effect of the intact LOC innervation in mice is to downregulate auditory nerve activity, as LSO lesions enhance amplitudes of auditory-evoked potentials. LOC neurons are like VE neurons known to be cholinergic (White and Warr, 1983; Darrow et al., 2006), but are also shown to release additional transmitters, such as dopamine and GABA, at the efferent-afferent synapses under the inner hair cells (Eybalin et al., 1993; Gaborjan et al., 1999; Maison et al., 2003). In

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combination with experiments from Liberman and colleagues, reporting a greater hearing loss after noise exposure in LSO-lesioned mice (Darrow et al. 2007), the functional role of LOC neurons is therefore hypothesized to be related to neuroprotection by noise-induced trauma.

LOC neurons have been studied *in vitro* in rat (Fujino et al., 1997; Adam et al., 1999) and mouse (Sterenborg et al., 2010). Residing within the LSO, LOC neurons can be distinguished from LSO principal neurons by their smaller size and by their electrophysiological properties of lower membrane potentials, higher input resistance, and spike trains with characteristic first spike delays during 200 ms depolarizing current steps, caused by outward A-type K^+ currents. Furthermore, as opposed to the principal neurons, LOC neurons lack the hyperpolarization-activated Ih current (Sterenborg et al., 2010). Based on this information, we were able to localize and record from LOC neurons for comparisons between the vestibular and olivocochlear efferent systems in Paper I.

1.2 AUDIO-VESTIBULAR AFFERENCE

A hallmark of central auditory processing is that the afferent information is split into parallel ascending pathways that process and extract different features of a sound. Another characteristic of the ascending auditory pathway is its multi-synaptic nature, being composed of at least four synapses compared to only one for vision. From the auditory nerve, the information travels via the cochlear nucleus (CN) to the SOC in the brainstem, continuing via the lateral lemniscal nuclei (NLL) to the inferior colliculus (IC) in the midbrain, passing the medial geniculate body (MGB) in the auditory thalamus, and finally to the auditory cortex (AC), where we ultimately perceive and understand what we hear (Fig 1.2). Afferent processing of sound is also strictly hierarchical, in a way that processing at each level of the pathway is essential for a complete and correct perception at cortex level. Thus, comprehensive understanding of low level auditory processing is, axiomatically, important in order to understand the underlying mechanisms to cortical sound representation. As low level processing in the auditory system is more complex than in other sensory systems (Winer and Schreiner, 2005), we still lack complete understanding of these brainstem circuits. For example, the binaural circuits underlying sound localization have been extensively studied in multiple species (reviewed in: Nishino and Ohmori, 2009; Grothe et al., 2010; Grothe and Koch, 2011). However, another critical function of the auditory system is to extract meaning from complex acoustic signals, such as animal vocalizations and human speech, which has been shown to rely primarily on monaural cues (Plomp, 1976). Paper II – IV are aimed at elucidating the mechanisms of such monaural auditory brainstem pathways.

1.2.1 Parallel processing streams arise in the cochlear nucleus – CN

Environmental sounds are usually highly complex, and our ears consequently receive waveforms comprised of all of the acoustic stimuli in the environment. Throughout the auditory pathway in the brain, intricate computations take place in order to extract biologically relevant information from the composite waveforms. Information then needs to be extracted regarding multiple properties of sound, such as pitch, intensity, localization, meaning and origin. Furthermore, in a process known as auditory streaming, the brain segregates sound information from multiple simultaneous sources, for example multiple speakers. These processes begin in CN, the first synaptic station in the brain. Each auditory nerve fiber, carrying frequency-specific sound signals, branches to make contact with several types of neurons in the CN. Although receiving the same input, the CN neurons display diverse computational functions due to cell type-specific molecular specializations, in combination with specific projection patterns.

Auditory spectro-temporal information is usually segregated into a dorsal and a ventral stream. Spectral information usually follows a dorsal stream, from the dorsal cochlear nucleus (DCN), with direct projections to the inferior colliculus (IC) in the midbrain. The DCN is the complex in a way that it is difficult to predict their output based on incoming auditory nerve stimulus. This is due to modulations by interneurons and extensive intrinsic mechanisms in the principal cells. Furthermore, the DCN cells do not preserve the temporal firing pattern of their inputs (Rhode et al., 1983), but are, however, believed to have a role in spectral sound localization (Oertel, 1991; Young et al., 1992; Spirou et al., 1993; May 2000).

Temporal information usually follows a ventral stream, from the ventral cochlear nucleus (VCN) to the IC via the SOC. Temporally precise neurons in the anteroventral cochlear nucleus (AVCN) are known for a role in temporal sound localization, where binaural projections to the LSO and the medial superior olive (MSO) enable computations of interaural level and time differences, respectively (reviewed by: Middlebrooks, 2015). Another prominent auditory brainstem nucleus that receives input originating in the AVCN is the superior paraolivary nucleus (SPON). SPON is activated primarily by monaural sound stimulation and is not believed to have a role in sound localization (Kuwada and Batra, 1999; Behrend et al. 2002; Dehmel et al. 2002; Kulesza et al. 2003; Kadner and Berrebi, 2008).
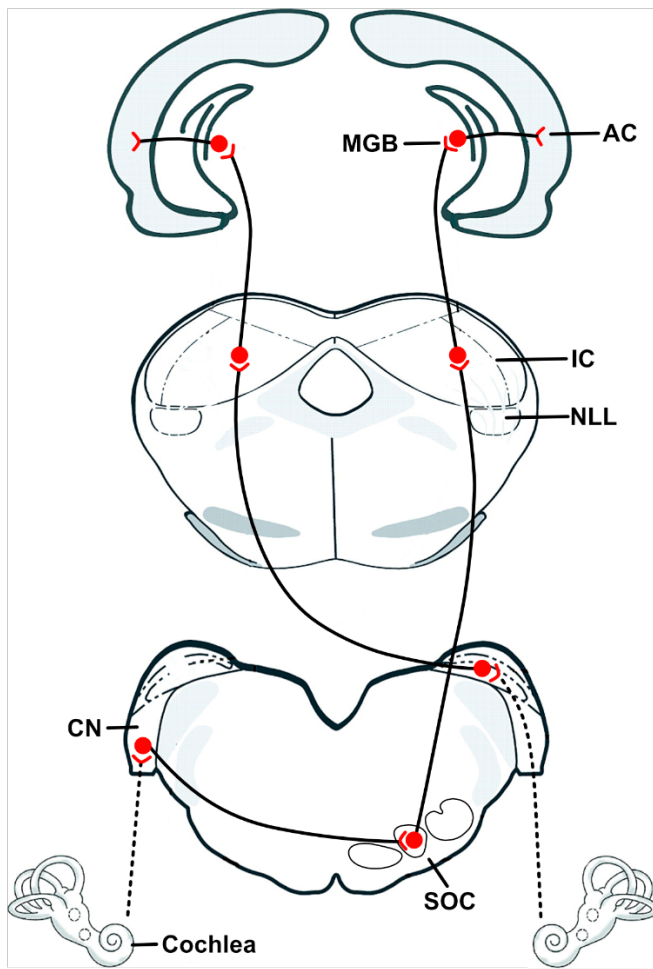
Figure 1.2. Schematic illustration of ascending parallel processing streams in the central auditory system. All auditory information from the inner ear is processed in the cochlear nucleus (CN). In CN the processing is divided into parallel streams. Some information (see right CN) follows a dorsal stream and directly innervates the inferior colliculus (IC) in the midbrain, while other information (see left CN) crosses the midline and bypasses the nuclei of the superior olivary complex (SOC) and the nucleus of the lateral lemniscus (NLL), from where IC receives projections. From IC the information is transmitted to the auditory cortex (AC) via the medial geniculate body (MGB) in the auditory thalamus.

1.2.2 Monaural temporal processing of sound in the superior paraolivary nucleus – SPON

SPON is a prominent structure (e.g. Kulesza Jr et al., 2002) that resides within the superior olivary complex (SOC) in the ventral auditory brainstem (Fig 1.2). SPON is conserved across mammalian species (for summary see Saldaña and Berebbi, 2000), including rhesus monkeys (Bazwinsky et al., 2005) and humans (Moore, 2000; Bazwinsky et al., 2003). In contrary to the surrounding SOC nuclei SPON is monaural, primarily receiving innervation originating in the contralateral CN (Cant and Benson, 2003; Fig 1.3). Most well-documented is the glycinergic input from the ipsilateral medial nucleus of the trapezoid body (MNTB; Moore and Caspary, 1983; Spangler et al., 1985; Helfert et al., 1989; Bledsoe et al., 1990; Kuwabara and Zook, 1991; Banks and Smith, 1992; Sommer et al., 1993; Schofield, 1994; Smith et al., 1998; Saldaña et al., 2009). This inhibition can be described as feed-forward since the MNTB is driven by a glutamatergic projection from bushy cells in the contralateral AVCN (von Gersdorff and Borst, 2002). The temporal precision of this signal is preserved by passing through the gigantic calyx of Held synapse (Schneggenburger and Forsythe, 2006) and then being converted to inhibition, which is relayed to SPON. However,

SPON has also been shown to receive excitatory input from the octopus cells in the contralateral posteroventral cochlear nucleus (PVCN; Friauf and Ostwald, 1988; Thompson and Thompson, 1991; Schofield, 1995; Saldaña et al., 2009). The octopus cells are intriguing as they are held to be the most timely precise neurons of the brain (e.g. Golding et al., 1999), but their exact role for auditory processing is still unclear. Therefore, it is important to investigate whether potential octopus cell drive of SPON is selective and strong enough to play a functional role.
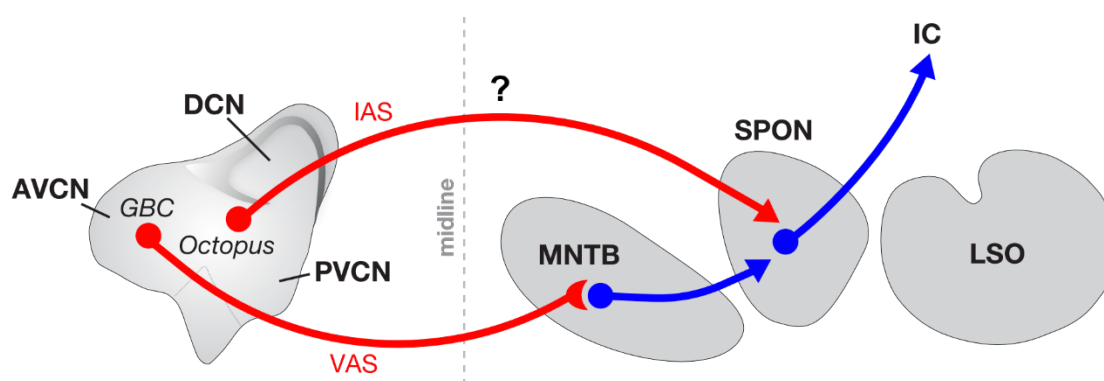

Figure 1.3. The SPON brainstem circuitry. SPON receives feed-forward inhibition from the MNTB, originating in a glutamatergic projection from globular bushy cells in the contralateral AVCN. SPON also receives excitatory input from the VCN, possibly only from the PVCN octopus cells, the physiological significance of which is unknown. SPON is GABAergic and its main output is a strictly ipsilateral projection to the IC in the midbrain, which is a main station for convergence of information from parallel auditory brainstem processing streams.

The first physiological characterization of SPON conducted *in vivo* was in bat (Groethe et al., 1994) and rabbit (Kuwada and Batra, 1999) and SPON responses to sound stimuli have since been investigated in gerbil (Behrend et al., 2002; Dehmel et al., 2002), as well as rat and mouse (Kulesza Jr et al., 2003; Kadner et al., 2006; Felix II and Berrebi, 2007; Kulesza Jr et al., 2007; Kadner and Berrebi, 2008). Results vary slightly between studies, but there is a general agreement that SPON shows little spontaneous activity and responds with timely precise offset responses to tones. Although less consistent, many studies also report an onset response to tones (Grothe, 1994; Kuwada and Batra, 1999; Behrend et al., 2002; Dehmel et al., 2002; Felix et al., 2013). Furthermore, SPON neurons show synchronous spiking during amplitude-modulated sounds, and best so at low modulation frequencies (<20 Hz), where they phase-lock faithfully and spike at each cycle (Kadner and Berrebi, 2008). The same study also reported an ability of SPON neurons to detect brief silent periods, or gaps, within an ongoing sound (Kadner and Berrebi, 2008). Based on these *in vivo* studies, SPON has been attributed a role in encoding coarse temporal information of sound, which is important for the ability to discriminate essential acoustic features contained in communication sounds.

In contrast to the binaural system, knowledge about the cellular mechanisms involved in monaural sound processing are less well underbuilt. With respect to SPON, nothing was known about the nature of its cellular or synaptic properties before the commencement of this thesis work. For instance, it was neither known what causes the prominent offset response to sounds *in vivo*, nor why SPON sometimes respond with an onset response, which implies that these neurons, in addition to their documented strong inhibitory drive (Kulesza Jr et al., 2003; 2007) are subjected to brief and strong excitation. These questions are important to resolve in order to understand the role of SPON for mammalian acoustic processing.

1.3 CELLULAR MECHANISMS FOR SENSORY PROCESSING

Neurons communicate with each other mainly through electrical events, or action potentials. A comprehensive understanding of the anatomical connections and basic electrophysiology of neurons within a neural system is therefore fundamental to appreciating its function and dysfunction. The integration and propagation of electrical events is modulated by the expression and maintenance of specific ion channels. Subtle cell type-specific differences in ion channel setup can have large effects on the excitability of a neuron and the pattern of action potential firing (e.g. Kaczmarek, 2012). Moreover, different neural systems face distinct challenges. For hearing and balance, the temporal acuity of neural processing is highly important as a functional vestibular system requires continuous and instant modulations, and the auditory system needs to accurately encode acoustic information with micro-second precision.

Most ion channels are built up of proteins that form narrow and highly selective pores across the otherwise impermeable plasma membrane of neurons. In humans there are more than 200 genes encoding pore-forming plasma membrane ion channels, and the ion channel setup is close to identical in mouse (Jegla et al., 2009). A single neuron typically expresses up to 10 different channels. Furthermore, multiple proteins (subunits) often heteromerize to form a functional pore. As a corollary, there are practically endless possibilities in ion channel combinations and structures, which is reflected by a large functional diversity.

Voltage-gated ion channels are common in the audio-vestibular brainstem. The pore of a voltage-gated channel is opened and activated by a conformational change in the three-dimensional configuration of the protein caused by a change in voltage (reviewed in Miceli et al., 2015). Voltage-gated channels that are commonly involved in regulating the integrative properties and electrical excitability of neurons include the Kv family of ion channels and hyperpolarization-activated nucleotide-gated (HCN) channels. The Kv family is a large, functionally diverse multi-subgroup family of K^+ channels. Their contribution to synaptic integration and excitability in the auditory brainstem is

reviewed by Johnston and colleagues (2010). In brief, low-threshold subgroups (e.g. Kv4, discussed in Paper I) only need small excitatory post-synaptic potentials (EPSPs) to open and therefore contribute to action potential thresholds or action potential firing in response to a brief stimuli. Furthermore, when partially active at rest they cause a hyperpolarization of the membrane potential in these neurons. The high-threshold subgroups need stronger depolarizations to open and are important for repolarization, thereby contributing to high-frequency firing and action potential width. The HCN channels are non-selective inward cation channels (reviewed by: Wahl-Schott and Biel, 2009; Lewis et al., 2010). There are four subunits (HCN1-4) with distinct kinetics, of which HCN1 and HCN2 are most common in the brainstem (Koch et al., 2004). They activate at hyperpolarized voltages, causing a typical sag voltage while counteracting the hyperpolarization and bringing the neuron closer to threshold. Thus, HCN channels are important for post-inhibitory rebound spiking (discussed in Papers II – IV). Similarly to some Kv channels, the HCN channels can be partially active at rest, but with a depolarizing effect on membrane potential. Furthermore, ion channels that are continuously open at rest, so-called leak channels, also contribute to resting membrane potentials, by allowing free diffusion of K^+ (or Na^+) across the membrane. Due to active Na^+/K^+ pumps, the inside of a neuron is hyperpolarized and K^+ rich compared to the outside. The net effect of leak channels is thus that K^+ moves out of the cell down its concentration gradient, giving a hyperpolarization of the resting membrane potential. Moreover, Ca^{2+} and Cl^- channels are also important in auditory brainstem signaling. For example, T-type and L-type Ca^{2+} channels contribute to rebound spiking and plateau potentials in the SPON (Paper II and IV), and the outward Cl^- channel KCC2, expressed in the SOC including SPON (Papers III and IV) and contributes to the inhibitory strength of MNTB signaling (Löhrke et al., 2005; Kopp-Scheinpflug et al., 2011).

An additional factor that affects synaptic integration and functional diversity in neurons is the subcellular localization of an ion channel. In general, action potential generation requires summation of multiple subthreshold inputs arriving at the dendrites of a neuron. Ion channel gradients over the somatodendritic axis can lead summation of inputs arriving to the distal and proximal dendrite, respectively. Also, passive dendritic filtering (ion leakage) gradually attenuates the amplitude and slopes of EPSPs as they travel along the dendrite. Filtering of dendritic inputs are especially important in SPON neurons, the dendrites of which can extend for several hundreds of microns (discussed in Paper III). Finally, synaptic integration is multifactorial and often highly complex, but knowledge of ion channel setup and subcellular expression patterns provides important clues as to a cell's integrative role.

2 AIMS OF THE STUDY

2.1 GENERAL AIM

To generate knowledge on cellular mechanisms and principles underlying afferent and efferent neural processing in the audio-vestibular system by using the mouse as a model.

2.2 SPECFIC AIMS

I. Record the cellular properties of the vestibular efferent neurons and compare them to those of the lateral olivocochlear neurons to shed light on their functional role at the inner ear sensory organs.

II. Clarify the cellular and molecular correlate of offset spiking to tones in the superior paraolivary nucleus.

III. Identify the origin of the excitatory input to the superior paraolivary nucleus.

IV. Investigate the development and function of superior paraolivary neurons in congenital deafness.

3 METHODOLOGICAL ASPECTS

Presented herein are the animal models and techniques utilized in Papers I – IV. Focus is laid on building an overall understanding of the basic methodology. For specific experimental and technical details, please see respective paper in Appendix I – IV.

3.1 ANIMALS AND MODELS

All experiments in Paper I – IV were performed in conformity with the rules set by the European Commission Council Directive (86/89/ECC) and approved by the local Swedish Animal Care and Use Committee (Permits N32/07, N13/10 and N52/13).

Paper I. The vestibular efferent neurons are cholinergic and therefore express the enzyme choline acetyltransferase (ChAT). A transgenic mouse model (Tallini et al., 2006) with enhanced green fluorescent protein (eGFP) knocked-in under a cholinergic locus which contains the ChAT and the vesicular acetylcholine transporter (VAChT) genes was therefore used. This way, all cholinergic neurons are highlighted and the vestibular efferent neurons could be localized under the microscope. For the same paper, wildtype Sprague Dawley rats were also utilized.

Paper II – III. Wildtype mice of C57BL/6 and CBA/CaJ strains were used.

Paper IV. A mouse model (Platzer et al., 2000) for congenital deafness bred on a C57BL/6 background was used to investigate the role of sensory deprivation on the superior paraolivary nucleus. The mouse model (α1D$^{-/-}$) has a functional knockout (KO) of the pore-forming alpha-subunit of the Cav1.3 ion channel. As this ion channel is expressed in the primary sensory cells in the auditory end organ, these mice are congenitally deaf. Results from this mouse were compared to those from wildtype mice.

3.2 GENOTYPING

3.2.1 PCR

Polymerase chain reaction (PCR) was utilized to ensure desired genotype for the transgenic mice studied in Paper I and Paper IV. The heterozygous ChAT-eGFP mouse was genotyped continuously and animals identified as positive for the eGFP insertion were utilized for experiments. The α1D$^{-/-}$ mouse was bred homozygously, and genotyping was thus not necessary on a continuous basis, but was performed upon arrival of the mice to the breeding facility.

The preparation of genomic DNA was performed by the HotShot method as described by Truett et al., 2000, with the adjustment that the final lysate was put through three freeze-thaw cycles before running the PCR, since this had a positive effect on the final outcome and reduced false negatives. Briefly, tissue samples were collected as tail-

snips. These were incubated for 40 min at 95 °C in an alkaline lysis buffer, quickly cooled to 4 °C, and thereafter briefly vortexed in a neutralizing buffer. Primers were utilized according to table 1 in Fig 3.1. Cycling conditions were chosen based on the specific primers in respective experiment. After PCR, samples were run on a 2 % agarose gel with 0.5 µg/mL ethidium bromide for 45 min at 100V (Fig 3.1).

Table 1	Primer 1	Primer 2
eGFP-ChAT	agtaaggctatgggattcattc	agttccacttgatgccgttc
α1D$^{-/-}$	tcttcggatggggttattga	gaccaacttctcagcccaac
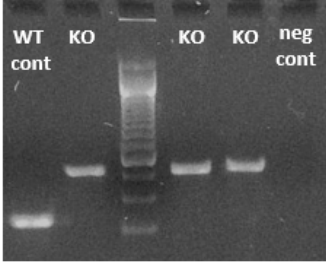
Figure 3.1. Table listing sense and antisense primers used for genetic identification of eGFP-ChAT mice and α1D$^{-/-}$ (KO) mice. Displayed on the right is one example of results from α1D$^{-/-}$ genotyping, where all three individuals were positive for the knockout.

3.3 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a classical neurobiological method based on the binding of specific antibodies to their respective epitopes in a biological tissue specimen.

3.3.1 Tissue preparation

Mice were deeply anesthetized with an overdose of sodium pentobarbital prior to trans-cardial perfusion with phosphate buffered saline (PBS) followed by ice-cold 4% formaldehyde in 0.1 M PBS. Brains were dissected, post-fixed for three hours (or overnight) in 4% formaldehyde at 4°C, and transferred to 30% sucrose in 0.1 M PBS at 4°C overnight. Coronal sectioning of the mouse brainstem (Fig 3.2) was performed at 30 µm thickness using a cryostat (Leica model CM3050S).
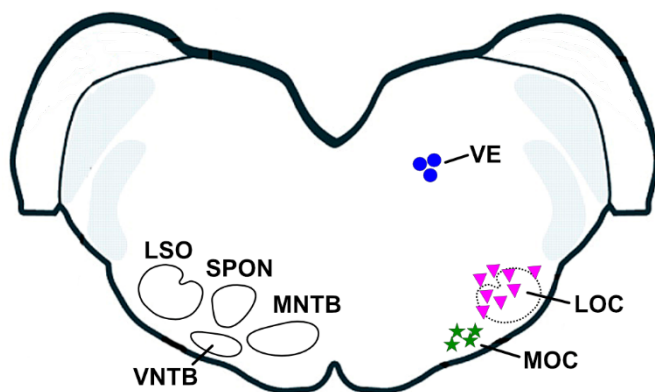

Figure 3.2. Schematic view of a coronal section of mouse auditory brainstem showing localizations of vestibular (VE) and olivocochlear (MOC/LOC) efferent neurons on the right and the superior olivary complex, with the superior paraolivary nucleus (SPON) on the left.

3.3.2 Antibody staining

For the staining procedure, sections were rinsed three times for 10 minutes in 0.1 M PBS and thereafter pre-incubated in blocking solution for one hour at room temperature. Sections were incubated in blocking solution overnight at 4°C with one or more specific primary antibodies (see respective Paper). The following day, sections were washed three times with PBS before a two hour incubation in darkness with secondary antibodies, conjugated to a fluorescent molecule, in blocking solution at room temperature. After a final wash in PBS, sections were mounted onto glass slides with an anti-fading medium and stored in darkness at -20°C until visualization.

3.3.3 Microscopy and image analysis

Visualization of immunolabeling and image acquisition was performed with fluorescence microscopes (BX6, Olympus; Zeiss Observer Z1, Carl Zeiss) equipped with cameras (XM10, Olympus; AxioCam MRm, Zeiss). Images were digitally processed using softwares including Photoshop CS5, ImageJ and AxioVision 4.8. When pixel intensity was investigated, it was measured in unprocessed monochrome images. A background measurement was obtained at corresponding areas in each image and set as 0 %. For some experiments confocal images, which give a higher resolution in the z-plane, were obtained with a LSM780 confocal microscope (Zeiss). Excitation lasers and recorded emission spectra were chosen based on the corresponding fluorophores for each antibody. Stacks of 16-bit grayscale images were obtained with axial distances of 100-500 nm between optical sections and pixel sizes of <50 nm at the maximum zoom factor. Representative images from one confocal plane were chosen and processed using AxioVision 4.8.

3.4 ELECTROPHYSIOLOGY

The excitability and intrinsic properties of neurons can be investigated using electrophysiological techniques that measure voltage and current changes on a cellular level. We use an *in vitro* technique called patch-clamp, where individual neurons are investigated in acute mouse brain slices. SPON neurons are large (20-30 µm) and multipolar, with 4-7 thick and long dendrites (Saldaña and Berrebi, 2000). This makes SPON a problematic target for patch-clamp, as the dendrites easily break during the slicing procedure. Also, SPON tissue is unusually "sticky" due to fibers running through the area, leading to an increased difficulty targeting the SPON after the fibers are being myelinated early during the postnatal development.

3.4.1 Slice preparation

Animals were deeply anesthetized with sodium pentobarbital and adequate depth of anesthesia was assessed by a negative withdrawal reflex. Following decapitation, the brainstem was carefully collected and placed in low-sodium, high-sucrose artificial cerebrospinal fluid (aCSF). Coronal brainstem slices containing the SOC or the VE were obtained at a thickness of 180–220 μm using a Vibratome and incubated at 32°C in normal aCSF for 30 min, after which they were allowed to cool to room temperature. aCSF was bubbled continuously with carbogen gas (95% O_2–5% CO_2), setting the pH to 7.4. For current-clamp and voltage-clamp recordings, a K-gluconate based internal pipette solution adjusted to pH 7.3 was used.
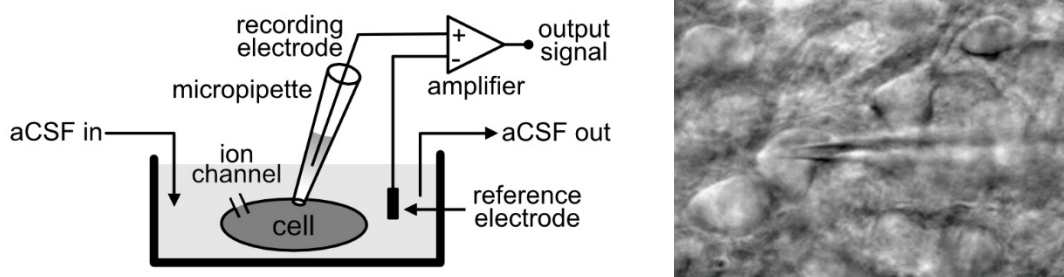

Figure 3.3. Schematic illustration of an *in vitro* patch-clamp setup. The brain slice is kept in continuously flowing artificial cerebrospinal fluid (aCSF) in a chamber under the microscope. When a target neuron is visually localized (see actual SPON neurons on right), an opening is made in the membrane to allow continuous flow between the inside of the cell and the inside of a micropipette. The electrode inside the pipette is connected to an amplifier which enables manual control over the voltage or current over the cell membrane.

3.4.2 Whole-cell patch-clamp

Slices were transferred to a recording chamber perfused (~3 ml/min) with oxygenated aCSF at room temperature (Fig 3.3 left). SPON principal cells were viewed with an upright microscope (Axioscope, Zeiss) equipped with a digital CCD camera (Orca 2, Hamamastu) using a 40x water-immersion objective (Zeiss Achroplan) and infrared-differential interference optics. The SPON cells were first visually identified (Fig 3.3 right) by their large somata in a defined area medial to the LSO and, subsequently, physiologically identified by their rebound spiking response to hyperpolarization (see Paper II). The VE neurons were localized by their eGFP expression. Neuron capacitance was estimated from the compensation measurement under voltage clamp. Whole-cell current- and voltage-clamp recordings were performed with a Multiclamp amplifier (700B, Molecular Devices) using borosilicate glass microelectrodes with a final tip resistance of 5-10MΩ for whole-cell patch-clamp recordings. During voltage-clamp recordings, the series resistance was compensated by 70-80%. Typically, basic

membrane properties were investigated by injection of 500 ms hyperpolarizing and depolarizing current steps of 20-100 pA intervals in current clamp, and current conductances in voltage-clamp by gradually stepping to lower voltages from a holding potential of -50 mV (Fig 3.4).
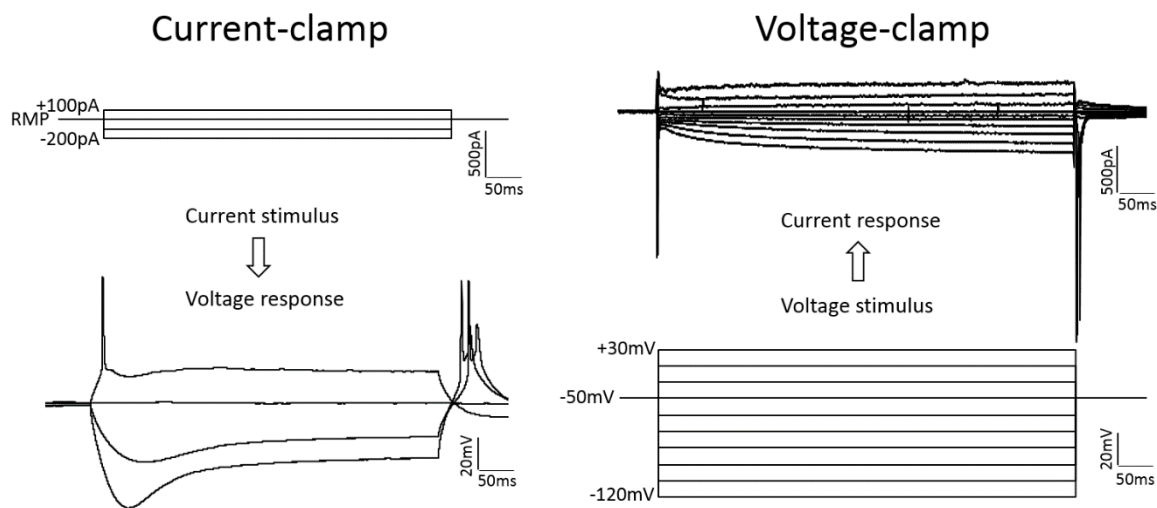

Figure 3.4. Illustration of typical basic stimuli for whole-cell current- and voltage-clamp, respectively. When a current is injected the voltage response (eg. action potential) is observed, and when the voltage is stepped to either more positive or negative voltages, the current conductances across the membrane is observed.

3.4.3 Data analysis

Data analysis of electrophysiological data was performed offline using softwares Clampfit (version 10.3) and IGOR Pro (version 6.35A5). The level of significance was tested using student's t-test if the data showed normal distribution. When data did not meet the criteria for normal distribution, non-parmetric Kruskal-Wallis was used within groups and Mann-Whitney was used for between group comparisons.

4 RESULTS AND COMMENTS

4.1 THE VESTIBULAR EFFERENT NEURONS

4.1.1 The vestibular efferent neurons were successfully localized in the ChAT-eGFP mouse

In Paper we investigated the VE neurons *in vitro*. One of the main reasons there is such limited number of studies on the intrinsic properties of VE neurons is because they are difficult to target in an *in vitro* preparation. They are very few in number and lack cytoarchitectural borders, making it close to impossible to visually identify the neurons in a brain slice. In Paper I we took advantage of the VE neurons being cholinergic and utilized a transgenic mouse (Tallini et al., 2006) that expresses the fluorescent protein eGFP in cholinergic cells. Verification of the eGFP expression pattern was made using immunohistochemistry against choline acetyltransferase (ChAT). The eGFP intrinsic to the mouse model was enhanced using an eGFP-targeting, GFP-conjugated antibody. We could confirm that VE neurons are positive for eGFP, while the OC efferent neurons are not (Fig 4.1).
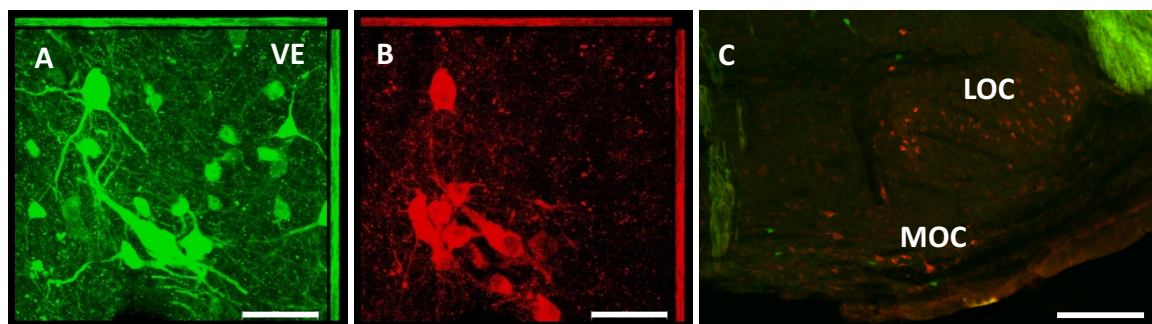

Figure 4.1 Efferent neurons in the transgenic mouse labeled against eGFP (green) and ChAT (red). (A-B) VE neurons are positive for eGFP and the labeling overlaps with ChAT. (C) Olivocochlear neurons do not express eGPF, but are labeled against ChAT. Scale bars: A-B 50 μm, C 200 μm. *Modified from Paper I (Figure 1).*

4.1.2 The vestibular efferent neurons show cellular properties distinct from auditory efferent neurons

VE neurons being positive for eGFP enabled us to successfully localize these neurons under the microscope and to perform the first electrophysiological recordings from VE neurons in *in vitro* brain slices. For comparisons, we also recorded from LOC neurons, which, although not positive for eGFP, could be identified in the LSO by their previously characterized intrinsic properties.

We performed whole-cell current- and voltage-clamp. Our results demonstrate that VE neurons, similarly to LOC neurons, are hyperpolarized (-76.5±2.7 mV), lack a voltage sag when injected with hyperpolarizing current, and respond with linear voltage current relationships in the hyperpolarizing range. In the depolarizing range, however, VE

neurons displayed intrinsic properties and firing patterns distinct from LOC neurons. For example, VE neurons showed smaller voltage deflections triggered by small depolarizing currents, which is in accordance with their significantly smaller input resistance (p<0.01). When injected with identical depolarizing stimuli, VE neurons consistently fired a single spike followed by a long inter-spike interval (Fig 4.2A), while LOC neurons display a long latency to the first spike (Fig 4.2B). Furthermore, VE neurons show smaller amplitudes and larger action potential widths (Fig 4.2C).
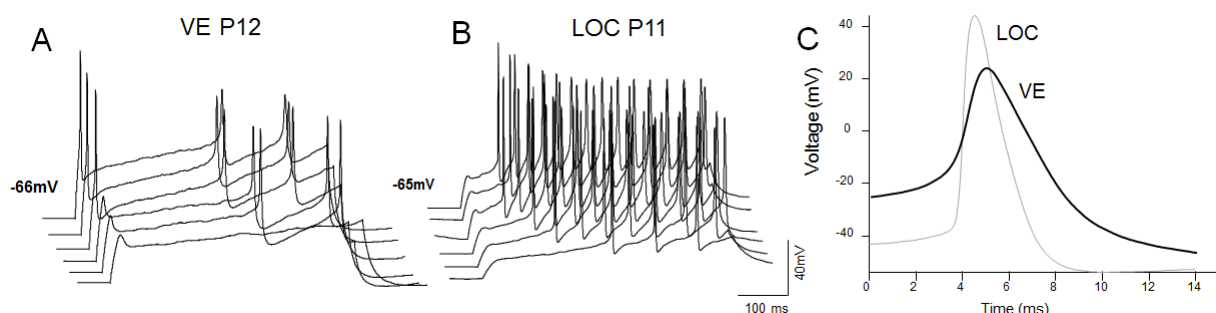
Figure 4.2 Action potential and response properties of efferent neurons to depolarizing current steps. (A) VE neurons respond with a single spike and a long first inter-spike interval. (B) LOC neurons respond with a long latency to first spike. (C) Averaged action potential shapes of LOC and VE neurons. *Modified from Paper I (Figure 3).*

4.1.3 The vestibular and auditory efferent neurons have Kv4 currents

In whole-cell voltage-clamp we were able to identify a transient outward current in both VE and LOC neurons (Fig 4.3A). The current amplitude was larger in VE neurons, but when examining the current density which takes neuronal size into account, this was smaller in VE neurons (Fig 4.3B). This is in accordance with VE neurons being larger in size than LOC neurons, as indicated by a significantly larger capacitance (p<0.01). Using immunohistochemistry, localizing the VE and LOC neurons by ChAT-labeling, we could confirm expression of Kv4.2 and Kv4.3 in both neuronal types (see Paper I).
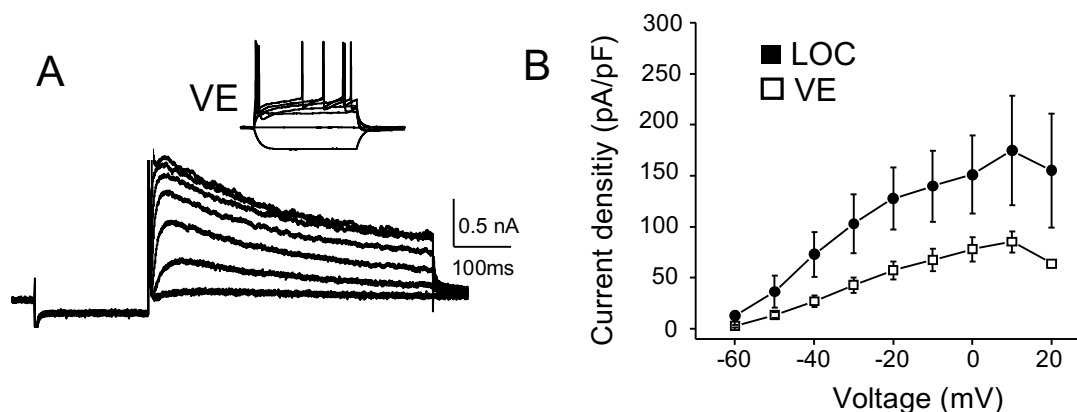

Figure 4.3 (A) Transient outward currents were recorded in VE and LOC neurons as exemplified by VE. (B) The current density was larger in LOC than in VE neurons, probably contributing to the lack of onset spiking in these neurons. *Modified from Paper I (Figure 6).*

4.2 THE SUPERIOR PARAOLIVARY NUCLEUS

4.2.1 Voltage-activated h currents and T-type Ca²⁺ currents have complementary roles for hyperpolarization-activated rebound spiking *in vitro*

In Paper II we recorded a post-inhibitory rebound in SPON neurons and further investigated what currents underlie this rebound response. SPON neurons display a voltage sag when injected with hyperpolarizing currents *in vitro*, which is indicative of the Ih current, an inward non-selective cation current. Accordingly, we recorded large inward currents in voltage-clamp mode that could be blocked with the specific blocker ZD7288 (Fig 4.4C-D). The expression of both HCN1 and HCN2 channel subunits, known to carry the Ih current (for reviews see: Rusznák et al., 2013; He et al., 2014), was confirmed with immunohistochemistry (Fig 4.4A-B).
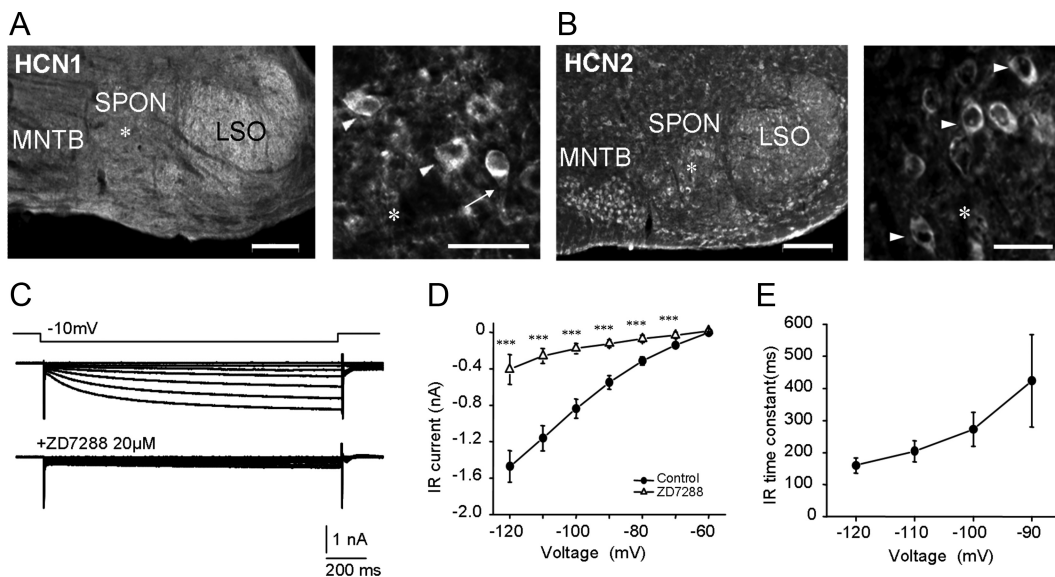

Figure 4.4. (A-B) Adult mouse auditory brainstem labeled against HCN1 and HCN2 channel subunits. (C) Inwardly rectifying current recorded in SPON neurons in voltage-clamp mode. The current could be blocked with Ih-specific blocker ZD7288. (D) Amplitudes the inward current with and without specific blocker. (E) Time constants of inward current at different voltages. ***=p<0.001. Scale bars in (A-B) are 200 μm in overviews and 50 μm in enlargements. *Modified from Paper II (Figure 2).*

An additional current recorded in SPON neurons in voltage-clamp was isolated investigated pharmacologically. This current was largely blocked by Mibefradil, which is a specific antagonist of T-type Ca²⁺ channels. Next we used the same pharmacological blockers in current-clamp to investigate their respective contributions to generating the rebound spiking. In some neurons blocking Ih abolished the rebound. In other cells blocking Ih altered the temporal precision of the otherwise very timely precise spiking (latencies <30 ms), indicating the Ih contributes to determining the rebound spiking latency (Fig 4.5A). Blocking low-voltage activated Ca²⁺ channels with

Ni^{2+} abolished the rebound spiking (Fig 4.5B). Furthermore, Mibefradil, which is specific for T-type LVA Ca^{2+} channels, also abolished the rebound spiking (Fig 4.5C). Together, these data indicate that Ih currents and T-type Ca^{2+} currents act in tandem to generate a precise post-inhibitory rebound firing in SPON neurons.
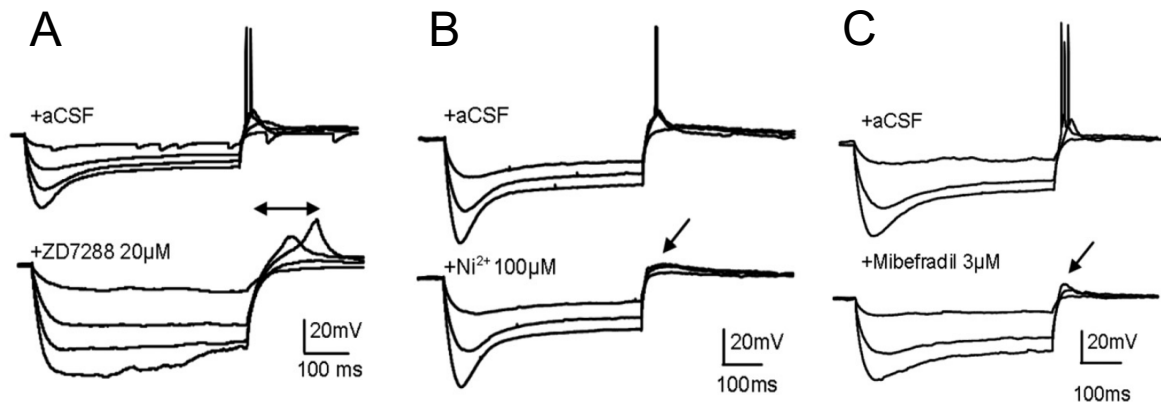

Figure 4.5. Current-clamp traces recorded in SPON neurons with the use of different pharmacological blockers. (A) Ih blocked by the Ih-specific blocker ZD7288 eliminates the voltage sag and affects the temporal precision of the rebound. General block of low-voltage activated Ca^{2+} currents with Ni^{2+} (B) or specific block of the T-type subgroup of low-voltage activated Ca^{2+} currents with Mibefradil (C) abolishes rebound spiking. *Modified from Paper II (Figure 4).*

4.2.2 Neurons of the superior paraolivary nucleus receive temporally precise excitatory input from octopus cells of the cochlear nucleus

In Paper III we investigated the origin of the excitatory input to SPON. Calretinin is a calcium-binding protein that has been shown to label octopus cells, the putative origin of the excitatory input, in cats (Adams, 1997). We therefore utilized immunolabeling against calretinin to reveal the intermediate acoustsic stria (IAS) projection from the PVCN to the SOC. To investigate the relationship between IAS fibers and the different SOC nuclei, we double-labeled against KCC2, which is highly expressed post-synaptically in SOC (Blaesse et al., 2006; Kopp-Scheinpflug et al., 2011). Calretinin clearly highlighted the IAS. The IAS, which is a projection known as difficult to visualize with tract tracing (e.g. Saldaña et al., 2009), branches upon reaching the ventral auditory brainstem, passes the MNTB without terminating on the principal neurons, and collateralize extensively in the SPON before bending rostrally (Fig 4.6A). KCC2 double-labeling show that SPON neurons receive rich innervation from thick calretinin-fibers (Fig 4.6B), whereas the LSO is devoid of calretinin fibers (Fig 4.6C). Furthermore, the octopus cell bodies were confirmed to stain positive for calretinin, as opposed to the globular bushy cells in the AVCN which receive calretinin-positive terminals but are devoid of staining within the cell bodies. Finally, a physiological approach was taken to investigate the quality and strength of the excitatory input to SPON. Since it is problematical to obtain dual recordings from the PVCN and the SPON in the same slice due to the trajectory of the IAS, we recorded spontaneously

released synaptic events that reflect all existing excitatory terminals impinging onto a SPON neuron. By applying a cocktail of pharmacological blockers, these miniature excitatory postsynaptic currents EPSCs (Fig 4.6D) were isolated, recorded and analyzed with respect to their properties. Since each synapse is unique in its properties (Branco and Staras, 2009), we used principal component analysis to seek evidence for or against multiple synapse properties. However, as no distinct clusters were observed in any of the cells (exemplified in Fig 4.6E), these results are more compatible with one homogenous class of excitatory input to SPON.
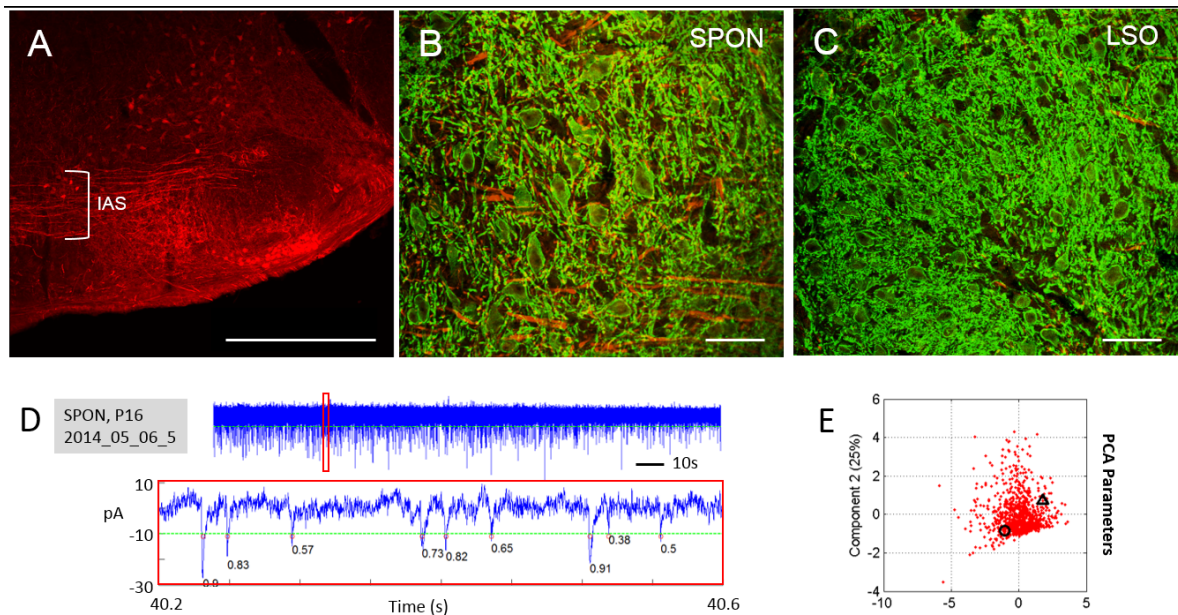

Figure 4.6. (A) Calretinin-positive fibers in the intermediate acoustic stria (IAS) branching into the contralateral SPON. (B-C) Double-labeling of calretinin (red) and KCC2 (green) showing rich calretinin innervation of SPON, while the LSO is devoid of calretinin labeling. (D) Spontanous EPSC recording and expanded time interval (bottom). (E) Principal component analysis (PCA) applied to mini-EPSC parameters indicate a homogenous input to SPON. *Modified from Paper III (Figure 1 and 3).*

4.2.3 In a congenital deafness model, the superior paraolivary nucleus display a staggered, but complete, prehearing development

In Paper IV we investigated early development of SPON neurons in a mouse model of congenital deafness (Platzer et al., 2000). Mouse hearing onset is around postnatal day 10 (P10). To investigate SPON in the deaf mouse before and after the age corresponding to normal hearing onset, data was collected from mice of ages P5-15, and was split up in three age groups, P5-8, P9-11 and P12-15. We first investigated the basic cellular properties of SPON neurons during hyperpolarizing current steps. Properties that are important for SPON rebound spiking were altered at the youngest ages. For example, the rebound rheobase was smaller (Fig 4.7D) and the area of the rebound was larger (Fig 4.7E). These properties normalized by age and reached wildtype levels after the age of normal hearing onset. The voltage sag was smaller throughout early development

(Fig 4.7C). The resting membrane potential, however, was similar in the deaf mouse compared to wildtype, but after the age of normal hearing onset SPON neurons from the deaf mouse were depolarized (Fig 4.7A). This might be explained by a decreased neuronal size at this age in the deaf mouse, as indicated by a decreased capacitance compared to wildtype (Fig 4.7B).
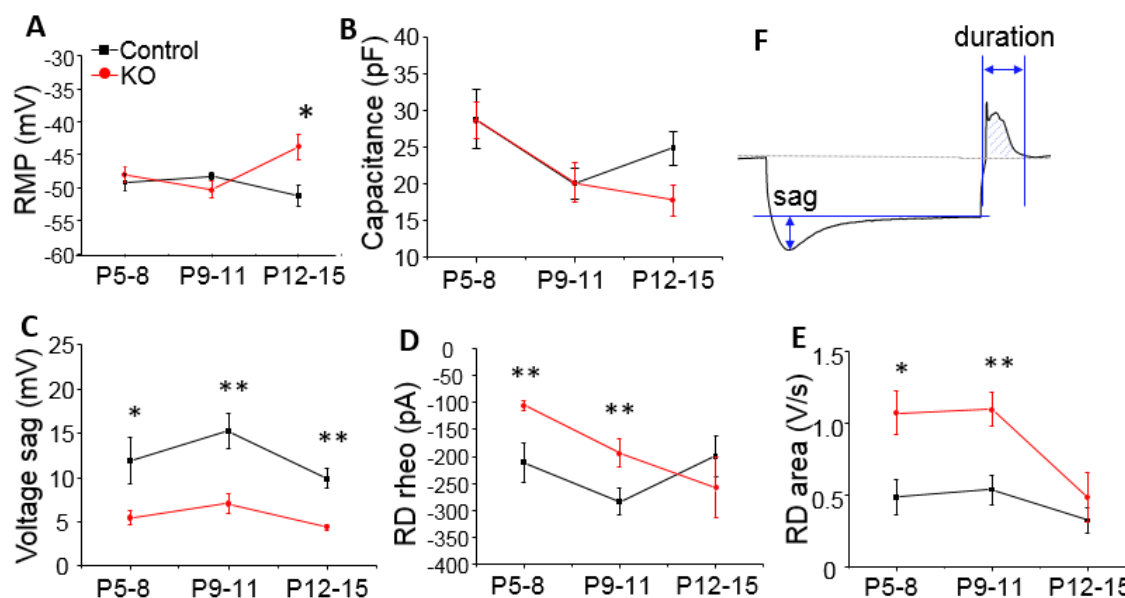

Figure 4.7. (A-E) Basic cellular properties of SPON neurons in deaf (red; KO) and normal hearing (black) mice of ages P5-8, P9-11 and P12-15. (F) Illustration showing how parameters in (C) and (E) were measured. *=p<0.05, **=p<0.01. *Modified from Paper IV (Figure 1)*.
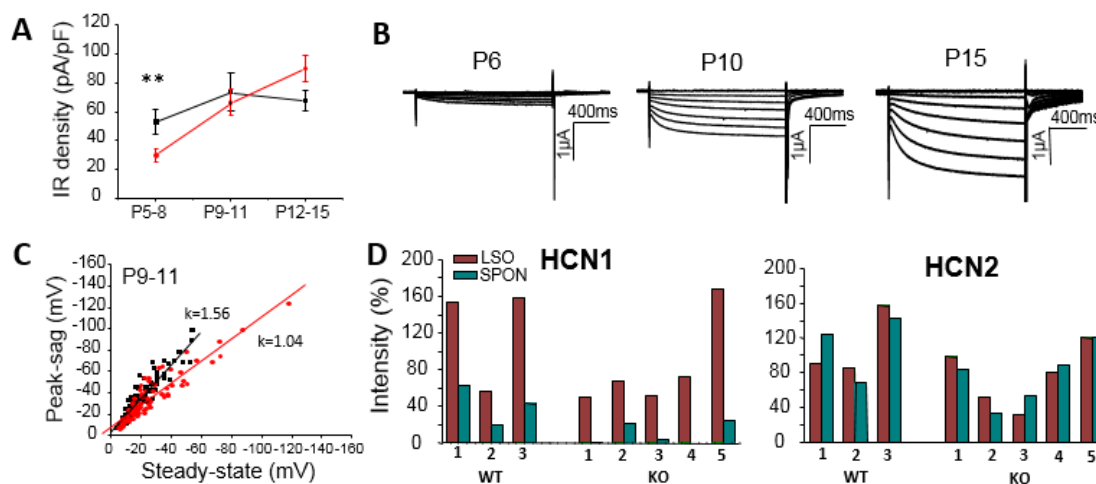

Figure 4.8. (A) Current density of inward current in SPON of deaf (red) and wildtype (black) mice in the three age-groups. (B) Voltage-clamp traces showing upregulation of inward current in example neurons representing each of the three age-groups. (C) Scatter plot of values for the peak voltage-sag against steady state-voltages at different hyperpolarizing current steps. (D) Pixel intensity measurements of HCN channel subunit immunolabeling in adult wildtype control (WT) and deaf (KO) mice. Intensity is presented as percentage above the background + 1 standard deviation. **=p<0.01. *Modified from Paper IV (Figures 2-3)*.

4.2.4 Ca^{2+} and h current homeostasis in combination with the neurotrophic factor neuritin contribute to the compensation in the sensory deprived superior paraolivary nucleus of congenitally deaf mice

In voltage-clamp mode we could identify an inwardly-rectifying current, which in Paper II was shown to consist of the Ih current, carried by HCN channels. The current was significantly smaller in deaf mice in the youngest age-group (P5-8; $p<0.01$), but was upregulated by age (Fig 4.8B). In fact, in the oldest age-group (P12-15), the current density was even overcompensated in neurons from the deaf mouse (Fig 4.8A). Furthermore, the current displayed less voltage-dependency in the deaf mouse at all ages, compared to wildtype (Fig 4.8 C). As an altered voltage-dependency indicates a change in the ratio of HCN channel subunits, we performed immunohistochemistry against HCN1 and HCN2. To get an estimation of the relative contribution of each subunit, pixel intensity was measured. This revealed a decreased HCN1 expression in the deaf mouse, while the HCN2 expression was stable (Fig 4.8D). As HCN2 is less voltage-dependent than HCN1, this is consistent with a decreased voltage-dependency in the deaf mouse, due to less HCN1 contributing to the current.

SPON rebound spiking can be divided into three subtypes (Fig 4.9A). When analyzing the subtype distribution over age in deaf and wildtype SPON neurons, it became evident that the complex spiking subtypes, especially the so-called plateau-spiking subtype, is overrepresented in the deaf mouse in the two youngest age groups (Fig 4.9B). This subtype distribution normalized over age, and reached close-to wildtype ratios in the oldest age group. (Fig 4.9B).
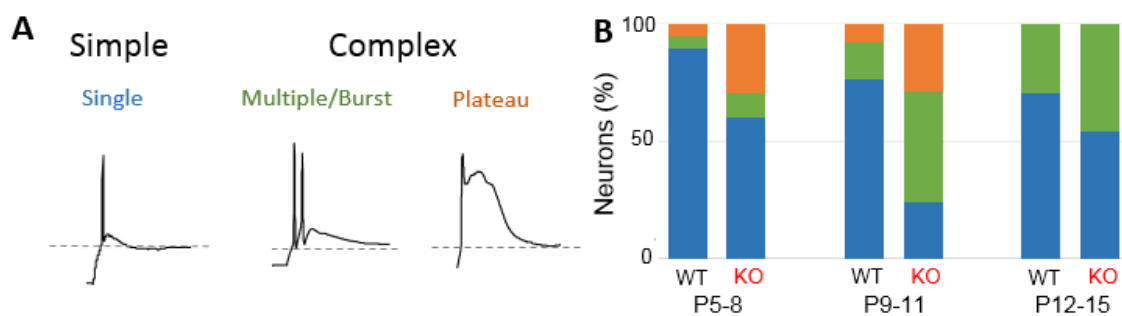

Figure 4.9. (A) There are three rebound spiking subtypes of SPON neurons: single-spiking, multiple/burst-spiking and plateau-spiking. (B) Subtype ratios in deaf (KO) and wildtype SPON. In wildtype the single spiking subtype is most prevalent throughout early postnatal development, but in deaf SPON, the complex spiking subtypes are overrepresented at early ages. *Modified from Paper IV (Figure 4).*

Finally, we investigated the expression of the neurotrophic factor neuritin in congenitally deaf mice as well as in normal hearing mice. Neuritin expression was found in several SOC nuclei, including the lateral superior olive (LSO), the MNTB and SPON. Neuritin expression was stronger in the congenitally deaf mice compared to in wildtype (Fig 4.10A-B). We further wanted to know if neuritin was localized pre- or post-synaptically. For this purpose, we performed double-immunolabeling between neuritin and either the pre-synaptic marker synaptophysin (Fig 4.10C-E) or the post-synaptic marker KCC2 (see Paper IV). Interestingly, neuritin labeling is found inside and along dendrites, surrounding the soma and extracellularly between cells, but does show much overlap with either of the markers.
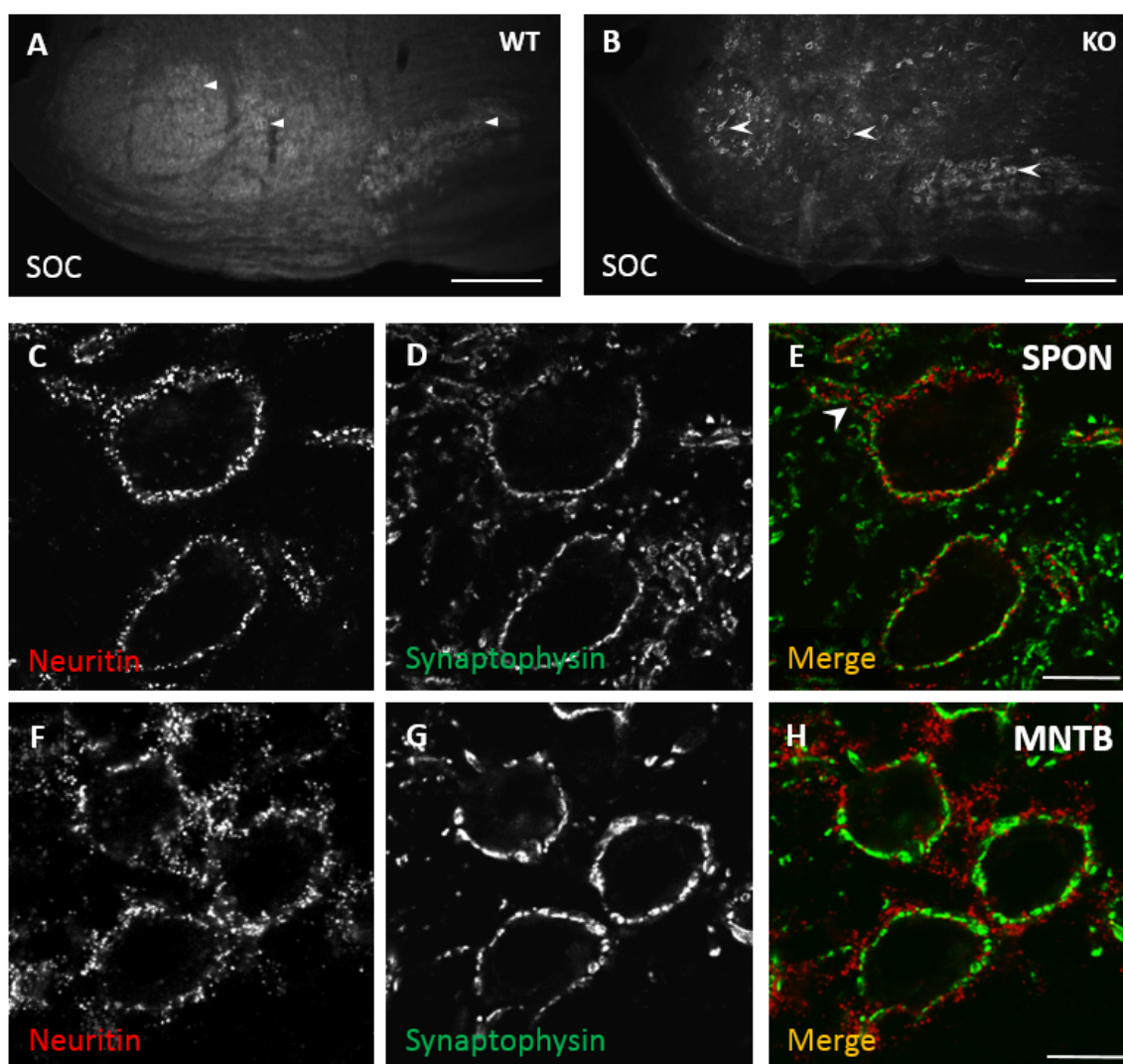

Figure 4.10. (A-B) Overviews of neuritin labeling in brainstem of young adult deaf (KO) and wildtype (WT) mice. (C-H) Confocal images of double-labeling of SPON or MNTB against neuritin and synaptophysin. Arrowheads in (A-B) indicate example-neurons positive for neuritin and arrowhead in (E) indicates neuritin puncta inside a SPON dendrite. Scale bars: (A-B) 200 μm, (C-H) 10 μm. *Modified from Paper IV (Figures 5-6).*

5 GENERAL DISCUSSION

5.1 VESTIBULAR EFFERENCE

Vestibular efference represents an old evolutionary adaptation to protect the peripheral sensory organ from damage by global shut-down during situations of sensory overload (Sienknecht et al., 2014). The addition of a dedicated auditory papilla in land vertebrates has separated the vestibular and auditory efferents and more sophisticated and specialized functions have developed (Sienknecht et al., 2014). For instance, the appearance of the outer hair cell cochlear amplifier has further separated the auditory efferents into the LOC and MOC systems, in which the latter supports the outer hair cell function (Rabbit and Brownell, 2011). Here, we compare the VE neurons to the LOC neurons, which both have been implied to modulate the firing rate of the afferent fibers in mammals (Goldberg and Fernández, 1980; Groff and Liberman, 2003), possibly by expression of neuromodulators (Scarfone et al., 1996; Maison et al., 2003; Darrow et al., 2006). We were, therefore, not surprised to find similarities between the VE and LOC neurons when comparing their cellular intrinsic properties. Both of them are characterized by low resting membrane potentials and delayed latency firing upon depolarization (Fujino et al., 1997; Adam et al., 1999; Sterenborg et al., 2010). The low membrane potential is partially due to absence of currents in the hyperpolarizing range, which indicates that, in contrast to the afferent neurons, these efferent neurons do not use inhibition to enhance their integrative function or produce rebound spiking (e.g. Paper II; although see Mathews et al., 2015). Another similarity was their robust expression of the Kv4 ion channels, which resulted in strong transient outward currents upon depolarization. This current will divert the membrane potential from the spike threshold and thereby shunt spiking (Storm, 1988). Due to geometric differences between VE and LOC neurons, the functional output of this potassium current generated specific firing patterns in the respective neuron type. The VE neurons are larger and therefore these Kv4 currents do not shunt the spiking response to the same degree as in LOC neurons, allowing a phasic response in addition to their delayed tonic firing. In other words, although the VE and LOC neurons have the same set of ion channels, their individual cell sizes influence how they respond to inputs. Based on their bimodal firing pattern, we speculate that the VE neurons have the capacity to generate both to phasic and tonic responses, which may promote their documented fast and slow effects on semicircular canal afferent fiber activity (e.g. Goldberg and Fernández, 1980). Their low cellular gain (i.e. small change in response to increasing current stimulation) may be suitable for providing tonic feed-back to the macular organs. Taken together, the cellular properties of VE neurons are compatible with the divergent efferent projections to all vestibular end-organs.

5.2 SPON IN AUDITORY AFFERENCE

A robust offset response to sound is well documented in SPON *in vivo* (Kuwada and Batra, 1999; Behrend et al., 2002; Dehmel et al., 2002; Kulesza Jr et al., 2003; Kadner et al., 2006; Felix II and Berrebi, 2007; Kulesza Jr et al., 2007; Kadner and Berrebi, 2008). This thesis demonstrates that SPON neurons *in vitro* consistently respond with a rebound spiking upon release from hyperpolarization (Paper II). In addition, we were able to abolish the rebound spiking both *in vivo* and *in vitro* by selectively blocking either one of two complementary ion currents (the Ih and LVA Ca^{2+} currents), thereby confirming a link between the novel *in vitro* results and previous *in vivo* findings. In parallel, another seminal study documented rebound spiking in SPON and came to the same conclusions regarding the ionic mechanism by combining the physiology with mathematical modelling (Kopp-Scheinpflug el at. 2011). Moreover, both these studies emphasize that strong summation of inhibition is needed to trigger a rebound spike (Paper II; Kopp-Scheinpflug el at. 2011). Long or strong inhibition allows further recruitment of Ih and LVA Ca^{2+} conductances, which enables the membrane potential to reach spike threshold when the stimulus ends. This is consistent with the MNTB providing SPON with continuous inhibition during sound stimulation *in vivo*, leading to absence of spiking during sound input and an offset response upon sound cessation (Kulesza et al., 2003).

In the third study, we identified a neural correlate for the onset response to sounds *in vivo*, which has previously been overlooked, although documented by several groups in many species (Grothe, 1994; Kuwada and Batra, 1999; Behrend et al., 2002; Dehmel et al., 2002; Felix et al., 2013). The onset was recognized as originating from a single excitatory neuronal group, namely the octopus cells in the PVCN, which are among the timeliest precise neurons in the brain (Golding et al., 1999; Oertel et al., 2000). Dendrites of octopus cells stretch over a large range of frequency bands (Golding et al., 1995) and the cells need strong, convergent inputs at multiple frequencies to respond (Oertel et al., 2000). A high threshold in octopus cells might explain why the SPON onset response is less consistent than the offset response. Moreover, octopus cells answer with high synchronicity and precision to broadband transients (Oertel et al., 2000) and formant-like sounds (Rhode, 1998) – stimuli which have not yet been tested in SPON. The combined results, thus, imply that the onset/offset response in SPON originate in excitation from octopus cells and feed-forward inhibition from MNTB, respectively. The MNTB inhibition to SPON originates in AVCN globular bushy cells, which are equipped to meet the extreme timing demands of binaural auditory processing (von Gersdorff and Borst, 2002). Since the main inputs to SPON consists of the two most temporally specialized pathways in the brain, we think it is reasonable to assume that SPON is involved in the processing of temporal information. The cellular properties

and mechanisms we find in SPON are consistent with a more relaxed temporal precision compared to binaural processing that demands microsecond resolution. The on-off spiking pattern combined with phase-locking to amplitude modulated sounds up to a few hundred Hz indicate that SPON would be suitable to encode robust fluctuations in sound energy over time with millisecond resolution. For instance, their onset response may be elicited by the upstrokes in the composite waveforms of animal vocalizations and human speech. Contrarily, SPON is inhibited during high energy intervals in the waveform, allowing time for the summation needed to elicit an instant transient response following a waveform down-stroke. The role of inhibition in the central auditory pathways that process timing information has been controversial. While some evidence suggests that precisely-timed and brief inhibition can sharpen coincidence detection of binaural excitation (Brand et al., 2002; Franken et al., 2015), it is clear from this thesis work that this is not the only way that inhibition works in brain stem circuits that process timing information.

The question is how the coding of the coarse temporal sound cues in SPON affects the processing at the next level. SPON is strictly GABAergic and all neurons participate in an ipsilateral projection to the IC (Mugnaini and Oertel, 1985; González-Hernández et al., 1996; Kulesza Jr and Berebbi, 2000; Saldaña and Berebbi, 2000; Saldaña et al. 2009). The IC is a major hub for convergence of the multiple parallel processing streams arriving from the auditory brainstem (Ehret and Merzenich, 1988). A plausible role is to increase the contrast between regions of high or low sound energy in ongoing acoustic stimuli, thus contributing to sculpting out acoustic attributes that are used for reconstructing the auditory environment into "auditory objects" (Shinn-Cunningham & Wang, 2008; Shamma et al., 2011). Indeed, a recent study by Felix II et al. (2015) demonstrates that pharmacological inactivation of SPON-derived inhibition shapes the responses to periodic stimuli in the IC through both direct and indirect effects.

In summary, our *in vitro* data is in accordance with a role for SPON in encoding a wide range of behaviorally salient sound attributes. Since SPON is an evolutionary conserved structure, the documented cellular properties and mechanisms in this thesis could have implications for how natural sounds are analyzed and encoded in the mammalian brain. This includes coarse temporal changes in the absolute energy over time, as found in animal vocalizations (Grimsley et al., 2011) and the speech waveform (Rosen, 1992).

5.3 SPON IN CONGENITAL DEAFNESS

Our hearing and balance contribute greatly to our everyday life. Their importance for our physiology is easily reflected in pathological conditions affecting these sensory systems, such as seasickness, dizziness and vertigo, as well as hearing impairments or deafness with sequential psychosocial consequences. Such conditions often have profound impact on our experienced quality of life.

Hearing loss is the most common birth defect and permanent bilateral sensorineural hearing loss affects about one out of every 500 newborns (Morton and Nance., 2006; Hilgert et al., 2009). As hearing plays a fundamental role for language development (Kuhl, 2010), children born with congenital deafness fail to develop adequate speech and language skills if they are left untreated. Nowadays, the leading deafness therapy is to surgically insert cochlear implants (CIs) into the inner ear that translate incoming sound to electrical pulse trains, which are transmitted to the brain via the cochlear nerve. Today, according to the American Food and Drug Administration (2012), the number of people that have a restored hearing thanks to this elegant technique exceeds 300.000 world-wide. However, although children fitted with CIs have good chances of acquiring near normal language skills (Svirsky et al., 2004), the outcomes of CI implantation for optimal acquisition of speech and language skills vary considerably, even if implanted within the recommended time frame (Lammers et al., 2015). Congenital deafness represents an extreme state of sensory deprivation, and such lack of auditory activity is known to affect the development of the central auditory system (Klinke et al., 1999). Knowing that the auditory brainstem, and more specifically SPON, is important for processing of communication sounds, one possibility is that the displayed variability in speech and language acquisition may arise from alterations in SPON. Interestingly, auditory brainstem response (ABR) waveforms exhibit slower and less precise neural activity both in CI-implanted patients (Gordon et al., 2008; Sparreboom et al., 2010; Lammers, 2015) and in congenitally deaf cats (Tillein, 2012). However, to the best of our knowledge, SPON has previously never been studied in sensory deprived animal models. To elucidate the role of sensory deprivation for the development and function of SPON, in Paper IV we compared the electrophysiological profile of SPON neurons in wildtype mice with those in a mouse congenital deafness model (Platzer et al., 2000).

Our findings show that SPON neurons in the deaf mouse have a staggered, but complete prehearing development. Not only are the neurons able to compensate for the lack of auditory input in time for normal hearing onset, but they also display a highly stereotypic development, with almost identical output function and signaling patterns as in wildtype SPON. Such stereotypic development suggests a possible contribution of a genetic program to the maturation of SPON, which then is activated independently

of auditory input. Due to the complexity of central sensory integration it is at this stage impossible to exclude that activity in another sensory system, for example vision, provides sufficient activity to drive the development. This could however be investigated by removing distinct sensory cues separately, for example by dark-rearing or removing the whiskers of a congenitally deaf mouse prior to experiments. Perhaps more likely to contribute to the compensatory mechanisms in the deaf SPON is the documented upregulation of the neurotrophic factor neuritin, possibly keeping the SPON neurons in a prolonged immature state, thereby avoiding unfavorable plasticity and promoting neuronal survival.

As for a prospective involvement of SPON in the inter-individual variability of speech and language in CI fitted children, our data contradict such hypothesis. Instead, our results suggest that SPON develops normally in deaf individuals, indicating that early implanted children should have comparable brainstem capacity for processing of communication sounds as normal hearing individuals, and that the outcome of an auditory prosthesis relies more on processing in higher order brain areas, beyond the level of the SPON.

6 ACKNOWLEDGEMENTS

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8   APPENDIX I – IV