GATA Factors Regulate Inner Ear Development and Midbrain Neurogenesis

Maarja Haugas

Department of Veterinary Biosciences
Faculty of Veterinary Medicine
and
Developmental Biology Program
Institute of Biotechnology
and
Viikki Doctoral Programme in Molecular Biosciences
University of Helsinki

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Thesis supervisor Docent Marjo Salminen
Department of Veterinary Biosciences
University of Helsinki
Finland

Pre-reviewers Docent Marja Mikkola
Institute of Biotechnology
University of Helsinki
Finland

Docent Kirsi Sainio
Institute of Biomedicine
University of Helsinki
Finland

Opponent Professor Annette Neubüser
Institute of Biology I
University of Freiburg
Germany

Custos Professor Jyrki Kukkonen
Department of Veterinary Biosciences
University of Helsinki
Finland

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This thesis is based on the following publications, herein referred to by their roman numerals (**I-V**).

I Lilleväli, K., **Haugas, M.**, Pituello, F. and Salminen, M. (2007) Comparative analysis of Gata3 and Gata2 expression during chicken inner ear development. *Dev Dyn.* **236**, 306-313.

II Lilleväli, K., **Haugas, M.**, Matilainen, T., Pussinen, C., Karis, A. and Salminen, M. (2006) Gata3 is required for early morphogenesis and Fgf10 expression during otic development. *Mech Dev.* **123**, 415-429.

III **Haugas, M.**, Lilleväli, K. and Salminen, M. Defects in sensory organ morphogenesis and generation of cochlear hair cells in *Gata3*-deficient mouse embryos. *Hear Res.* In press.

IV **Haugas, M**., Lilleväli, K., Hakanen, J. and Salminen, M. (2010) Gata2 is required for the development of inner ear semicircular ducts and the surrounding perilymphatic space. *Dev Dyn.* **239**, 2452-2469.

V Kala, K., **Haugas, M.**, Lilleväli, K., Guimera, J., Wurst, W., Salminen, M. and Partanen, J. (2009) Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. *Development.* **136**, 253-262.

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Abstract

The zinc-finger transcription factors GATA2 and GATA3 in vertebrates belong to the six-member family that are essential regulators in the development of various organs. The aim of this study was to gain new information of the roles of GATA2 and GATA3 in inner ear morphogenesis and of the function of GATA2 in neuronal fate specification in the midbrain using genetically modified mouse and chicken embryos as models.

A century ago the stepwise process of inner ear epithelial morphogenesis was described, but the molecular players regulating the cellular differentiation of the otic epithelium are still not fully resolved. This study provided novel data on GATA factor roles in several developmental processes during otic development. The expression analysis in chicken suggested that GATA2 and GATA3 possess redundant roles during otic cup and vesicle formation, but complementary cell-type specific functions during vestibular and cochlear morphogenesis. The comparative analysis between mouse and chicken *Gata2* and *Gata3* expression revealed many conserved aspects, especially during later stages of inner ear development, while the expression was more divergent at early stages. Namely, expression of both *Gata* genes was initiated earlier in chicken than mouse otic epithelium relative to the morphogenetic stages. Likewise, important differences concerning *Gata3* expression in the otic cup epithelium were detected between mouse and chicken, suggesting that distinct molecular mechanisms regulate otic vesicle closure in different vertebrate species. Temporally distinct *Gata2* and *Gata3* expression was also found during otic ganglion formation in mouse and chicken.

Targeted inactivation of *Gata3* in mouse embryos caused aberrant morphology of the otic vesicle that in severe cases was disrupted into two parts, a dorsal and a ventral vesicle. Detailed analyses of *Gata3* mutant embryos unveiled a crucial role for GATA3 in the initial inner ear morphogenetic event, the invagination of the otic placode. A large-scale comparative expression analysis suggested that GATA3 could control cell adhesion and motility in otic epithelium, which could be important for early morphogenesis. GATA3 was also identified as the first factor to directly regulate *Fgf10* expression in the otic epithelium and could thus influence the development of the semicircular ducts. Despite the serious problems in the early inner ear development, the otic sensory fate establishment and some vestibular hair cell differentiation was observable in pharmacologically rescued *Gata3-/-* embryos. Cochlear sensory differentiation was, however, completely blocked so that no auditory hair cells were detected.

In contrast to the early morphogenetic phenotype in *Gata3-/-* mutants, conditional inactivation of *Gata2* in mouse embryos resulted in a relatively late growth defect of the three semicircular ducts. GATA2 was required for the proliferation of the vestibular nonsensory epithelium to support growing of the three ducts. Concurrently, with the role in epithelial semicircular ducts, GATA2 was also required for the mesenchymal cell clearance from the vestibular perilymphatic region between the membranous labyrinth and bony capsule.

The gamma-aminobutyric acid-secreting (GABAergic) neurons in the midbrain are clinically relevant since they contribute to fear, anxiety, and addiction regulation. The molecular mechanisms regulating the GABAergic neuronal development, however, are largely unknown. Using tissue-specific mutagenesis in mice, GATA2 was characterized as a critical determinant of the GABAergic neuronal fate in the midbrain. In *Gata2*-deficient mouse midbrain, GABAergic neurons were not produced, instead the *Gata2*-mutant cells acquired a glutamatergic neuronal phenotype. Gain-of-function experiments in chicken also revealed that GATA2 was sufficient to induce GABAergic differentiation in the midbrain.

Abbreviations

APAF	apoptotic peptidase activating factor
ASCL	achaete-scute complex homolog (*Drosophila*)
ATOH	atonal homolog (*Drosophila*)
BDNF	brain derived neurotrophic factor
bHLH-O	basic Helix-Loop-Helix-Orange
BMP	bone morphogenetic protein
BOR	branchio-oto-renal
CDKN	cyclin-dependent kinase inhibitor
C-ZF	carboxy-terminal zinc finger
DLX	distal-less homeobox
E	days of embryonic development
EN	engrailed
Eph	Eph receptor
EYA	eyes absent homolog (*Drosophila*)
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FOG	Friend of Gata
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GATA	GATA binding protein
GBX	gastrulation brain homeobox
GJB	gap junction protein, beta
HDR	hypoparathyroidism, sensorineural deafness and renal disease
HELT	Hey-like transcription factor (zebrafish)
HES	hairy and enhancer of split (*Drosophila*)
HH	Hamburger and Hamilton stages in chicken
HMX	H6 homeobox
HOX	homeobox
Hz	hertz; unit of frequency; number of acoustic vibrations per second
Il	interleukin
IsO	isthmic organizer
JAG	jagged
KCNJ	potassium inwardly-rectifying channel, subfamily J
LFNG	lunatic fringe
NEUROD	neurogenic differentiation
NEUROG	neurogenin
NKX	NK transcription factor related (*Drosophila*)
NR4A3	nuclear receptor subfamily 4, group A, member 3
NTN	Netrin
N-ZF	amino-terminal zinc finger
OTX	orthodenticle homolog (*Drosophila*)
PAX	paired-box gene
POU3F4	POU domain, class 3, transcription factor 4
PROX	prospero-related homeobox
PRRX	paired related homeobox
PTF	pancreas specific transcription factor
Rb	retinoblastoma
SER	serrate

SHH	sonic hedgehog
SIX	sine oculis-related homeobox homolog (*Drosophila*)
SLC26A4	solute carrier family 26, member 4
SOX	SRY-box containing gene
SPP	Secreted phosphoprotein
TBX	T-box transcription factor
Th2	T-helper type 2 cell
TLX	T-cell leukemia, homeobox
WNT	Wingless-related
ZF	zinc finger

The symbols of genes and protein are written according to the International Committee on Standardized Genetic Nomenclature for Mice to the current nomenclature of Jackson laboratories (http://www.informatics.jax.org/mgihome/nomen/).

1 Review of the literature

1.1 Functions of the ear

Hearing is one of the most important senses in living beings, allowing interaction with the world around them. The process of hearing is accomplished by the ear, which is comprised of the outer, middle, and inner ear in mammals. Each part of the ear has a specific purpose in hearing process. The outer ear, which includes the pinna, the auditory canal, and the tympanic membrane collects and channels sound waves to the middle ear. Sound waves cause the tympanic membrane to vibrate and sequentially cause vibrations of the middle ear ossicles (malleus, incus, and stapes). These connected middle ear bones amplify and strengthen the vibrations and the sound waves pass from the middle ear through the oval window of the inner ear. Physical vibrations cause the fluid inside the inner ear auditory system, the cochlea, to move. This movement stimulates the sensory receptor cells (hair cells) that change the fluid movement into electrical impulses, which are sent through the cochlear portion of the VIII cranial ganglion to the primary auditory cortex in the temporal lobe of the cerebral cortex (Menner, 2003).

In humans the auditory system is capable of distinguishing sound frequencies from 20 Hz to 20,000 Hz. Vertebrates, however, are capable of hearing lower and higher frequencies. For instance, elephants can detect frequencies as low as 15 Hz and bats can detect those as high as 100,000 Hz. The frequency range of dog hearing is approximately 40 Hz to 60,000 Hz depending on the breed of the dog. Part of the reason many animals can hear better than humans is that their outer ears have more mobility and the shape of the outer ear may also aid in more proficient hearing. Most birds' hearing is at its most sensitive between 1000 Hz and 5000 Hz (Fay and Popper, 1994).

The inner ear also includes sensory organs for balance. Three types of structures, semicircular ducts, utricle, and saccule are involved in generating balance by providing information to the brain about the movement of the head: up and down, side to side, and tilting from one side to the other. As the head moves, sensory hair cells in the vestibular sensory organs send nerve impulses to the brain by the vestibular portion of the VIII cranial ganglion. These nerve impulses are processed in the brainstem and cerebellum (Ganong, 2005).

1.2 Inner ear structure

The inner ear is a three-dimensional membranous labyrinth encapsulated in a bony otic capsule closely following the forms of the labyrinth (Noramly and Grainger, 2002). The inner ear is composed of two functional parts. The dorsal vestibular division functions in detecting gravity and balance, while the ventral portion includes the auditory system for hearing. The diverse functions are carried out by various sensory organs in specific inner ear compartments. Each sensory organ contains sensory hair cells that transduce vibration or movement of the inner ear fluid into electrical impulses that are perceived by the brainstem.

1.2.1 The vestibule and auditory compartments

The vestibule comprises three orthogonally positioned semicircular ducts (superior, posterior, and lateral), the utricle, and the saccule. The vestibular portion of the inner ear is highly conserved across vertebrates usually consisting of two ventral sacs and three dorsal semicircular ducts, except for lamprey with two ducts and hagfish with only one duct (Mazan et al., 2000; Riley and Phillips, 2003).

In contrast to the vestibule, the auditory portion can vary greatly across vertebrates. Mammalian and avian hearing systems include a cochlear duct, whereas fish and amphibians lack cochlea, and the saccule has developed into an auditory structure (Fay and Popper, 2000). Furthermore, lagena is an auditory structure in fish, while it functions as a vestibular organ in birds and amphibians, and is missing entirely in mammals. Frogs have two additional hearing structures, the basilar and amphibian papillae (Figure 1; Riley and Phillips, 2003).

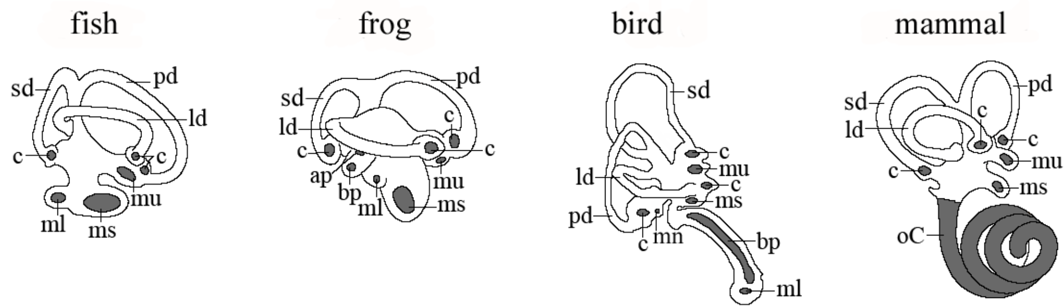

Figure 1. Schematic drawing of inner ears of different species. The vestibular system is highly conserved in fish, amphibians, birds, and mammals. The auditory portion is significantly different across vertebrates. The hearing system in birds and mammals lays in the cochlear duct, but fish and frogs lack cochlea and the saccule has developed into an auditory structure. In fish, the additional hearing organs are the macula lagena, and in frogs the basilar and amphibian papilla. Gray areas mark auditory and vestibular sensory regions. The endolymphatic duct is not shown. ap, amphibian papilla; bp, basilar papilla; c, crista; ld, lateral semicircular duct; ml, macula lagena; mn, macula neglecta; ms, macula sacculi; mu, macula utriculi; oC, organ of Corti; pd, posterior semicircular duct; sd, superior semicircular duct.

1.2.2 Inner ear fluids

The lumen of the membranous labyrinth contains endolymph, a specialized fluid with an unusual ionic composition (high K^+ and low Na^+), which is essential for sensory function. Highly specialized secretory cells, called marginal cells, in the stria vascularis of the mammalian cochlea and in tegmentum vasculosum of the avian cochlea produce endolymph. Vestibular secretory cells, dark cells, lie adjacent to the sensory epithelia and also produce endolymph (Torres and Giráldez, 1998; Hara et al., 2002; Ciuman, 2009). The circulation of the endolymphatic fluid and its pressure within the labyrinth are regulated by the endolymphatic sac and duct, which are connected to the utricle (Salt and Rask-Andersen, 2004).

Between the bony capsule and the membranous labyrinth is a space containing perilymph, which includes high levels of Na^+ and low levels of K^+. The perilymph is similar to the cerebrospinal fluid, and is formed locally from blood plasma by transport mechanisms or is an ultrafiltrate of the cerebrospinal fluid (Kellerhals, 1979).

The endolymph and perilymph are responsible for conducting sound vibrations coming from the outer and middle ears, they also respond to mechanical changes associated with body position and head movement (Shulman and Goldstein, 2006).

1.2.3 Sensory organs

The number of sensory organs involved in balance and auditory functions varies across vertebrates from three in hagfish to six in mammals, eight in birds and frogs, and nine in certain species of limbless amphibians (Fritzsch et al., 2002). Mammalian sensory organs include cristae at the end of each semicircular duct, maculae in the utricle and saccule, and the

organ of Corti in the coiled cochlear duct. In addition to the semicircular duct cristae, and utricular and saccular maculae, birds have a macula neglecta close to the posterior crista, and a macula lagena at the tip of the cochlear duct for balance sense (Figure 1; Swanson et al., 1990).

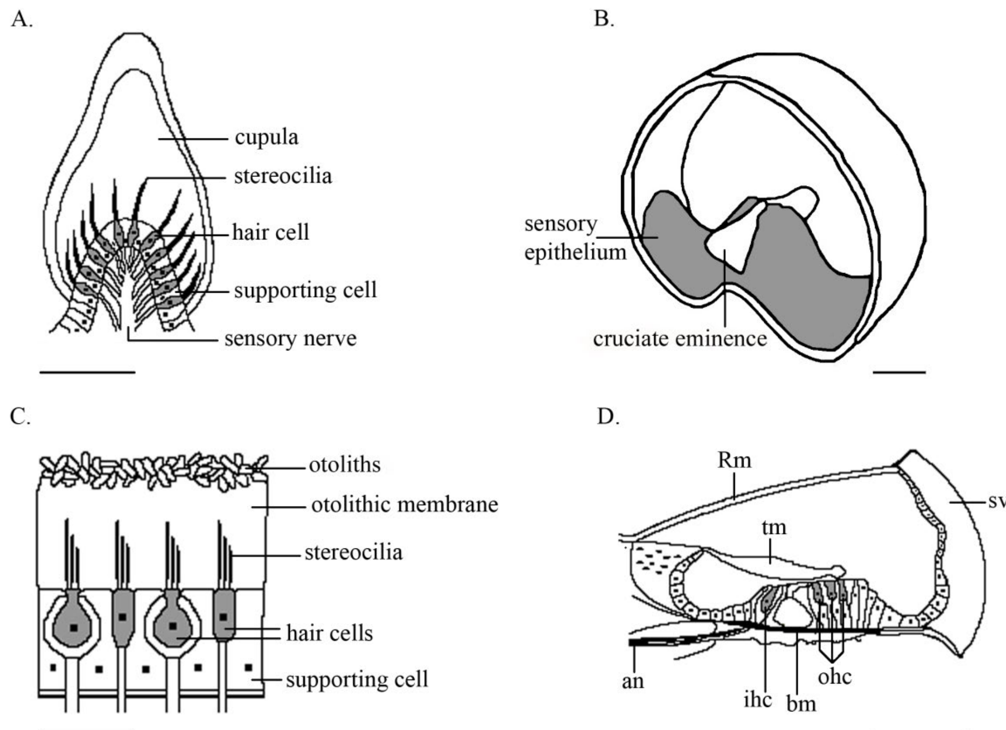

Figure 2. The structure of sensory organs in mammals. The schematic representation of (A) crista with hair and supporting cells, (B) sensory and nonsensory (cruciate eminence) epithelium of the crista in ampulla, (C) cellular organisation of the macula, (D) cross section through the organ of Corti. Gray areas mark sensory hair cells in A, C, and D, or sensory epithelium in B. an, afferent nerve; bm, basilar membrane; ihc, inner hair cell; ohc, outer hair cells; Rm, Reissner's membrane; sv, stria vascularis; tm, tectorial membrane. The scale bar: 50µm (A, D), 100µm (B), 20µm (C).

In general, mammalian and avian sensory functions are performed by cristae, maculae, and the auditory sensory organ, all of which comprise specialized cells involved in the sensory functions (hair cells) and others with nonsensory functions collectively called supporting cells:

a) The crista is located in an enlargement of the semicircular duct, called ampulla, situated close to the utricle and detects angular or rotational acceleration. Crista has an elongated ridgelike form overlaid by a conical glycoprotein layer called a cupula, which extends across the ampulla and establishes contacts with its opposite wall (Figure 2A). The stereocilia on the sensory cells lengthen into the cupula where they bend according to the endolymph movement in the semicircular ducts (Figure 2A). In mammals and birds, the superior and posterior crista include a nonsensory structure, the cruciate eminence, which divides the sensory region into two equal halves (Figure 2B; Desai et al., 2005).
b) The macula is a sensory organ of the utricle and saccule, responding to linear acceleration. The cellular layer of the macula is covered by a gelatinous mass associated with dense calcium carbonate crystals, otoliths, which transmit accelerational forces into the hair cell cilia (Figure 2C; Ballarino et al., 1985; Kido et

al., 1993). The macula is divided into lateral and medial parts by a region called striola, which determines the direction of hair cell polarity either away from (in the saccule) or towards the striola (in the utricle). The striola curves within the macula causing the hair cells to be sensitive to linear motion in multiple trajectories (Denman-Johnson and Forge, 1999; Jaeger et al., 2002).

c) In mammals, the cochlear sensory organ is called the organ of Corti, while in birds it is the basilar papilla. These auditory sensory organs are located over a movable basilar membrane and covered by a fibrous tectorial membrane that contacts the stereocilia bundles of the hair cells and vibrates due to sound wave movements (Figure 2D; Goodyear and Richardson, 2002). The auditory organ is composed of two types of hair cells and several specialized supporting cells in both mammals and birds. The avian basilar papilla has a regular mosaic pattern of tall and short hair cells, whereas in the organ of Corti the hair cells are organized into four arrays: one row of inner hair cells and three rows of outer hair cells (Goodyear and Richardson, 1997; Podgorski et al., 2007).

1.2.4 Structure and function of hair cells

Hair cells are named according to the bundle of hairlike protrusions that project from their apical surfaces. Ordered arrays of actin filaments containing microvilli, called stereocilia, and a single true cilium, the kinocilium form hair bundles (Figure 2A,C). The stereocilia of vestibular hair cells are arranged in rows of increasing length with the longest locating adjacent to the kinocilium (Shin et al., 2005; Kelley, 2006). Mammalian cochlear hair cells, in contrast, lose the kinocilium during development. The hearing organ displays hair bundles with shorter stereocilia located in the high-frequency sensing region and taller ones in the low-frequency sensing region (Hackney et al., 1993).

The hair bundle of a hair cell is directionally sensitive, therefore depending on the direction in which the stereocilia are deflected, cation channels spend more or less time in the open state. When deflection occurs towards the tallest stereocilium, cations (mostly K^+) from the endolymph rush in through the gates making the hair cell membrane potential more positive. This in turn activates voltage gated calcium channels at the basal end of the hair cells, leading to an influx of Ca^{2+} ions and an increase in the release of excitatory neurotransmitters. These neurotransmitters cause action potential in bipolar neurons of the VIII cranial ganglion. The action potentials travel through the afferent cochlear nerve into the cerebral cortex (Carey and Amin, 2006).

1.2.5 Supporting cells

Hair and supporting cells form highly organized patterns in inner ear sensory organs, so that one hair cell is always surrounded by several nonsensory supporting cells. The differentiation of supporting cells depends on the correct differentiation of hair cells, since mouse mutants with disrupted hair cell differentiation also show abnormal morphology of supporting cells (Erkman et al., 1996; Bermingham et al., 1999).

The vestibular sensory organs only contain one type of supporting cells, whereas the mammalian cochlear sensory epithelium has several highly specialized cell types, including pillar, Deiters', phalangeal, Hensen's, and Claudius' cells. For instance, the pillar cells develop between the inner and outer hair cells and form the tunnel in the organ of Corti, and Hensen's cells constitute the outer border of the organ of Corti (Barald and Kelley, 2004).

An important role for supporting cells seems to be the secretion of extracellular structures, like the tectorial membrane in the cochlea, the otolithic membranes in utricle and saccule, and the cupula in ampullas (Lim and Rueda, 1990; Riley and Grunwald, 1996).

1.3 Inner ear development

The morphology and cellular composition of the inner ear in humans is highly similar to that described in rodents, and thus the mouse serves as a remarkable animal model for investigating human ear development and disorders. The morphogenetic events of the human inner ear were described in detail in the beginning of 1900s (Streeter, 1906), whereas the molecular mechanisms controlling inner ear development are now, 100 years later, still not entirely understood. Great progress, however, has been made in understanding the genetic control of inner ear development during the last two decades mainly due to the advances in mouse gene targeting methodology (Capecchi, 1989; Rajewsky et al., 1996).

The correct morphogenesis of the inner ear involves a balance of cell proliferation, differentiation, survival, and death. These processes are tightly regulated by a network of extrinsic signals and intrinsic factors. Extrinsic signals are derived from tissues in the vicinity of the otic epithelium such as neural tube, notochord, ectoderm, pharyngeal endoderm, mesoderm, migratory neural crest cells, and periotic mesenchyme. On the other hand, intrinsic factors are expressed in the otic epithelium itself (Whitfield and Hammond, 2007).

1.3.1 Otic induction

The cranial non-neural ectoderm in vertebrates gives rise to all sensory placodes contributing to sense organs and cranial ganglia. At early neurula stages, this cranial ectoderm is called the preplacodal region (Bailey and Streit, 2006). Each sensory placode has a characteristic position in the embryonic head. The otic placode forms on both sides of the developing hindbrain just anterior to the level of the first somite (Torres and Giráldez, 1998; Begbie and Graham, 2001).

Otic induction is mediated by the localized action of signaling molecules derived from surrounding tissues. The first signals to initiate inner ear induction in the otic competence containing (preplacodal) ectoderm come from the underlying endoderm and mesoderm. During the neurulation stage, neural signals from the presumptive hindbrain completes inner ear induction that ends with the specification of a part of the preplacodal ectoderm to form the otic placode. Targeted mutagenesis in mice has not revealed any single genes preventing otic induction when inactivated, suggesting that inner ear induction needs a combination of synergistically acting factors (Torres and Giráldez, 1998; Anagnostopoulos, 2002; Chatterjee et al., 2010). Fibroblast Growth Factor (FGF) and Wingless-related (WNT) signals may direct the earliest steps towards inner ear induction (Groves and Bronner-Fraser, 2000; Baker and Bronner-Fraser, 2001; Ohyama et al., 2007; Schimmang, 2007).

1.3.1.1 Signaling molecules of FGF and WNT families

In vertebrates, the most prominent otic-inducing factors belong to the FGF family. FGF8 has been postulated as the first signal initiating inner ear development in both mouse and chicken (Ladher et al., 2005). In chicken, the endodermally derived FGF8 is required to initiate *Fgf19* expression in the mesoderm, which in turn is able to activate the expression of *Fgf3* in the mesoderm and neural tissue that directs ectodermal cells towards otic fate (Ladher et al., 2000; 2005). A similar sequence of events in otic induction seems to work in mouse embryos as well. Loss-of-function experiments in mouse demonstrate that FGF8 is required redundantly with FGF3 for otic induction (Ladher et al., 2005; Zelarayan et al., 2007). Combined loss of *Fgf8* and *Fgf3* in either the mesoderm or endoderm provided information that the major source of FGF8 for mouse otic induction is the mesoderm (Domínguez-Frutos et al., 2009). The presence of both FGF3 and FGF8 is essential for the normal level of FGF10 in the mesoderm (Ladher et al., 2005) and from there FGF10 acts redundantly with the hindbrain derived FGF3 to initiate the mouse otic placode formation (Alvarez et al., 2003;

Ladher et al., 2005; Zelarayan et al., 2007). FGF signaling is essential to establish a mitotically active progenitor domain and to activate *Pax2* expression in presumptive placodal ectoderm. Subsequently, FGF signaling becomes downregulated guiding otic progenitors out of the cell cycle and transit cells to a committed inner ear fate. The otic commitment, however, requires additional actions from the canonical WNT signaling, which mediates signal transduction through β-catenin (Alvarez et al., 2003; Wright and Mansour, 2003; Ladher et al., 2005; Freter et al., 2008). The essential role of WNT signaling has been confirmed in chicken, where mesodermal FGF19 induces *Wnt8c* expression in the hindbrain and these two factors synergize to induce the otic placode (Ladher et al., 2005). Ohyama and colleagues (2006) showed that WNT signaling mediates a fate decision between otic placode and epidermis in mice. The WNT pathway forces *Pax2*-expressing cells to differentiate into otic placode cells that start to express early otic-specific genes such as *Dlx5* and *Pax8*. On the other hand, cells that do not receive WNT signaling in the *Pax2*-expressing ectoderm are directed to an epidermal fate and express epidermal-specific gene such as *Foxi2*.

1.3.2 Early morphogenesis – from placode to otic vesicle

The induced otic placode is a one-layer thickened ectoderm, which becomes morphologically detectable lateral to the hindbrain rhombomeres 5 and 6 at approximately 8-10-somite stage in both chicken and mouse embryos (Figure 3; Anniko and Wikstrom, 1984; Alvarez and Navascués, 1990). The placodal ectoderm proliferates extensively and bends inwards, or invaginates, forming a cup-like structure (Figure 3; Torres and Giráldez, 1998).

The driving forces behind the invagination process are largely unknown. The otic placode invagination may, however, occur passively due to the pressure from the surrounding tissues instead of an active process driven by the cytoskeleton such as described during the optic and nasal placode bending (Legan and Richardson, 1997; Pilot and Lecuit, 2005). The otic epithelium also comes into intimate contact with the basal lamina of the neural tube, and this contact might have a key role in the progress of invagination, especially in chicken (Brown et al., 1998; Moro-Balbás et al., 2000; Visconti and Hilfer, 2002).

Once the otic placode invagination is complete, the extremities of the placode come together at the anterodorsal point to form the closed otic vesicle (Figure 3; Brigande et al., 2000a). The events and molecules driving the closure are largely unknown. Programmed cell death in the area that connects the surface ectoderm to the otic epithelium, however, is crucial for the proper closing of the otic vesicle in mice (Cecconi et al., 2004). The otic vesicle epithelium becomes separated from the surface ectoderm by means of differential expression of multiple types of cadherins like E-cadherin, N-cadherin, and P-cadherin (Nose and Takeichi, 1986).

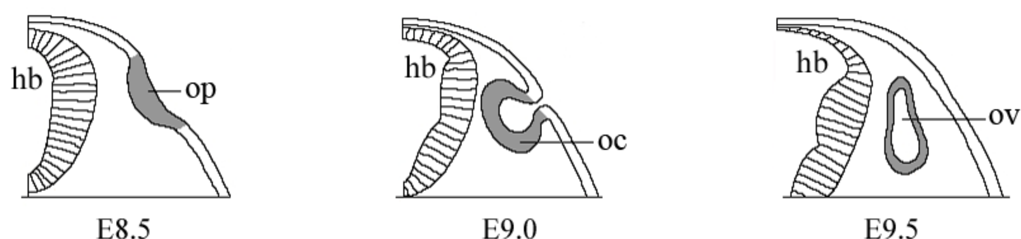

Figure 3. Formation of the otic vesicle. The otic placode (op) arises next to the hindbrain (hb), it invaginates to form the otic cup (oc) that closes into the otic vesicle (ov) and pinches off from the surface ectoderm. Gray areas mark the otic epithelium.

1.3.2.1 Patterning of the otic epithelium: intrinstic factors

Formation of the otic axes, or patterning of the inner ear epithelium starts at the placodal stage. Transplantation experiments of otic vesicles demonstrate that the axial polarity of the otic epithelium is established gradually. The anteroposterior axis is defined first, thereafter mediolateral, and finally the dorsoventral axis (Wu et al., 1998). This sequential process appears to be a general phenomenon in inner ear development, although the exact timing differs for different species.

Otic patterning may depend on a compartment-boundary model, where regionalized intrinsic gene expression domains divide the otic epithelium into lineage-restricted compartments that in turn determine the position and identity of different ear structures (Brigande et al., 2000b). This model has been verified by fate mapping experiments in chicken, which revealed that cells do not mix across an observed mediolateral boundary at the otic cup stage (Brigande et al., 2000a). Furthermore, altered expression of any of the regionalized genes causes defects in the specific inner ear structure derived from that region (Kiernan et al., 2002).

According to the model, the outgrowth of the endolymphatic duct in chicken is specified by boundaries between two gene expression domains (Brigande et al., 2000b). The hypothesis could be also valid in mice, since the endolymphatic duct does not develop in apoptosis-deficient embryos, where the otic vesicle is not able to close and thus the borders of different compartments cannot meet and induce the endolymphatic duct outgrowth (Cecconi et al., 2004). Brigande et al. (2000b) showed that the location of the sensory epithelia may also be specified by gene expression boundaries.

1.3.2.2 Patterning of the otic epithelium: hindbrain derived signals

Otic epithelium rotation experiments with respect to surrounding tissues suggests that in addition to intrinsic factors, extrinsic signals are also required for the specification and fixation of otic axes (Wu et al., 1998; Bok et al., 2005). The most important extrinsic influence to inner ear development comes from the closest hindbrain rhombomeres, 4 to 6, from which three main signaling pathways, WNT, FGF, and Hedgehog (HH), direct the axial patterning of the otic epithelium (Schneider-Maunoury and Pujades, 2007). Mutations in genes expressed in the hindbrain and those that cause defects in hindbrain segmentation and lead to secondary defects in inner ear development, have confirmed the importance of hindbrain signaling in otic patterning (Anagnostopoulos, 2002).

Hindbrain provides the major dorsoventral axial information to mouse and chicken inner ears (Bok et al., 2005). Essential secreted molecules come from the WNT family and are expressed in the hindbrain being responsible for the induction of a subset of dorsal otic genes. WNT and Bone morphogenetic protein (BMP) factors are involved in restricting the ventralizing effects of the Sonic hedgehog (SHH) (Riccomagno et al., 2002; Bok et al., 2005; Riccomagno et al., 2005; Fritzsch et al., 2006).

The formation of the other axes (anteroposterior, mediolateral) has remained less clear. The anteroposterior polarity of the otic epithelium may be controlled by the boundary between rhombomeres 5 and 6. This theory suggests that Eph receptors and ephrin ligands, which have mutually exclusive expression patterns between hindbrain segments, are involved in forming the anteroposterior polarity of the otic epithelium (Brigande et al., 2000b). Bok et al. (2005) showed that switching the anteroposterior orientation of the rhombomeres 4 to 7 before this axis is fixed, however, had no effect on inner ear anteroposterior axial patterning in chicken. The otic epithelium may acquire medial identity through WNT and FGF signaling (Bok et al., 2007). The hindbrain-derived FGF3 can play a key role in specification of the medial axis (McKay et al., 1996).

1.3.3 Later morphogenesis: from otic vesicle to membranous labyrinth

"Sculpting" of the otic vesicle into a complex membranous labyrinth takes place during mid-gestation in a relatively short time. All the main morphological structures are evident at E13.5 in mouse and by stage 30, according to Hamburger and Hamilton (HH), in chicken (Morsli et al., 1998; Bellairs and Osmond, 1998). Subsequent development mainly involves growth of the vestibular and cochlear structures, and cellular differentiation. Growth of the labyrinth is spatially controlled by properly localized cell proliferation and cell death. In addition, mesenchymal-epithelial interactions play an important role in inner ear morphogenesis (Martin and Swanson, 1993; Bissonnette and Fekete, 1996).

1.3.3.1 Formation of the endolymphatic duct and sac

The dorsal portion of the otic cup gives rise to the future endolymphatic duct. The endolymphatic duct is the first structure, which bulges out from the dorsomedial part of the otic vesicle close to the closure point at E10.75 in mouse and at HH23 in chicken (Bissonnette and Fekete, 1996; Morsli et al., 1998). Cecconi et al. (2004) showed that the vesicle closure event is particularly important for the outgrowth of endolymphatic duct. Thereafter, the duct elongates dorsally into a hollow tube and enlarges at its distal end to form a sac. In contrast to other mature inner ear structures, the endolymphatic duct and sac are not surrounded by the perilymphatic space or the bony capsule.

As mentioned earlier, the formation of the endolymphatic duct largely depends on signals coming from the adjacent hindbrain. The anteroposterior boundary within the endolymphatic duct is aligned with the boundaries between rhombomere 5 and 6, and expression of *Fgf3* in these hindbrain segments is especially important for the development of endolymphatic duct. Thus, the *Fgf3* knockout mice lack the endolymphatic duct and the ear expands into a large cyst because it fails to appropriately drain endolymphatic fluid (Mansour et al., 1993).

A normal endolymphatic function is important for hearing and changes in the endolymphatic structures are known to cause deafness in humans. For example, patients that suffer from Ménière's disease have vertigo, tinnitus, and hearing loss due to the reduced endolymphatic sac, which increases the volume of endolymphatic fluid in the labyrinth (Schmalbrock et al., 1996). Pendred syndrome, on the other hand, is characterized by profound deafness often associated with enlarged endolymphatic duct and sac (Naganawa et al., 2004).

1.3.3.2 Formation of the semicircular ducts

Morphogenesis of the three semicircular ducts is a complex remodeling process that can be divided in four critical steps (Figure 4). First, the otic vesicle epithelium grows out as a two-layered pouch of the dorsal otic vesicle at E10.5-11.5 in mouse and HH25 in chicken (Bissonnette and Fekete, 1996; Morsli et al., 1998; Cantos et al., 2000). The vertical pouch gives rise to the superior and posterior ducts, while the lateral pouch develops into the lateral duct (Figure 4A,C). In the next step, the epithelial layers in the middle of the pouches near each other so that cells of the opposite walls intercalate and form a temporary fusion plate structure at E11.5-12.5 and HH27 (Figure 4B,C). Then, the fusion plate cells are removed and three intact tubular semicircular ducts are formed at E12.5-13.5 and HH28 (Figure 4D,E; Martin and Swanson, 1993; Fekete et al., 1997; Kobayashi et al., 2008). In the fourth step, the semicircular ducts grow to their final form and size (Martin and Swanson, 1993; Fritzsch et al., 2006).

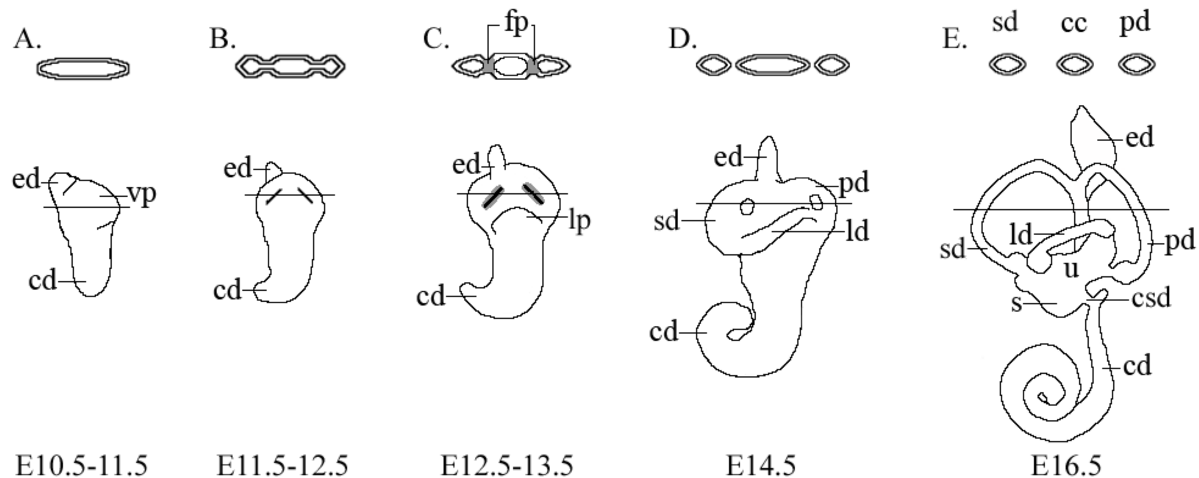

Figure 4. Schematic drawings of the developing mouse inner ear. The four-step development of semicircular ducts can be described: (A) the two-layered epithelial pouch grows out from the otic vesicle; (B-C) the epithelial layers in the middle of the pouch approach each other and form a fusion plate; (D) the fusion plate cells are removed; (E) the tubular semicircular ducts grow to their final size and form. Cross sections through the prospective superior and posterior semicircular ducts at the level of the lines are shown above. cc, common crus; cd, cochlear duct; csd, cochleo-saccular duct; ed, endolymphatic duct; fp, fusion plate; ld, lateral semicircular duct; lp, lateral pouch; pd, posterior semicircular duct; s, saccule; sd, superior semicircular duct; u, utricle; vp, vertical pouch.

Formation of the individual semicircular ducts is independently controlled by specific sets of transcription factors, since targeted mutagenesis of different factors show selective defects in one or two ducts (Fekete, 1999). For instance, inactivation of *Dlx5* results in the loss of the superior and posterior ducts, while the lateral duct is only reduced (Acampora et al., 1999). In contrast, *Otx1* is especially important for the development of lateral semicircular duct in the mouse (Morsli et al., 1999). The lateral semicircular duct is a unique feature of jawed vertebrates and it is missing in jawless organisms (lampreys), which also lack *Otx1* expression (Tomsa and Langeland, 1999). The appearance of the lateral semicircular duct in vertebrate evolution is theoretically a consequence of functional diversifications and duplications of an ancestral *Otx* gene (Mazan et al., 2000). Knockdown of the OTX1 function in zebrafish embryos results in an inner ear similar to lampreys (Hammond and Whitfield, 2006).

1.3.3.2.1 Molecular control of the epithelial pouches outgrowth

The pouch outgrowth in the dorsal otic epithelium is the first step in forming semicircular ducts. An interesting study showed that the apoptosis-deficient *Apaf1* mouse mutants develop strongly reduced semicircular ducts and demonstrated decreased proliferation in the semicircular ducts. Programmed cell death and proliferation often occur in same locations in the otic epithelium and the study proposed that cell death may induce cellular proliferation in the neighboring cells needed for the proper outgrowth of duct pouches by increasing locally the amount of growth factors (Cecconi et al., 2004).

FGF10 signaling has also been shown to be crucial for the normal semicircular duct outgrowth. Chang et al. (2004) used a fate mapping experiment in chicken and identified genesis zones for semicircular ducts adjacent to prospective cristae, which promote the outgrowth of the ducts. They showed that FGF10 secreted from the sensory region induces genesis zones possibly by activating *Bmp2* expression. This data is consistent with *Fgf10* null

mutants, which have reduced superior and lateral semicircular ducts and lack of the posterior one (Pauley et al., 2003).

Correct development of the epithelial semicircular ducts is also dependent on signals derived from the surrounding mesenchyme. This is illustrated by the inactivation of both *Prrx1* and *Prrx2* genes in the periotic mesenchyme that causes loss of the lateral semicircular duct and leads to the generation of thickened vertical ducts due to incomplete outgrowth of duct pouches (ten Berge et al., 1998).

1.3.3.2.2 Regulation of the fusion plate formation and clearance

Once the bilayered pouches have grown out from the dorsal vestibular epithelium, the two opposing walls of these pouches draw near each other, the basement membrane becomes disrupted and the cells lose their epithelial morphology. Next, the two layeres become fused in the center and form a fusion plate. Formation of the fusion plate and disruption of the basement membrane are particularly important for the correct development of tubular semicircular ducts. Mutations in genes regulating the above-named processes could result in either lack of the duct(s) or reduced duct(s).

The study by Salminen et al. (2000) demonstrated that a laminin-related axon guidance molecule, Netrin 1 (NTN1), also has an unexpected role in fusion plate formation. Secreted NTN1 protein is essential for the disruption of the fusion plate epithelium from the basement membrane that may occur by a receptor-independent manner through a direct interference of NTN1 on the laminin network beneath the fusion plate epithelium (Salminen et al., 2000; Matilainen et al., 2007). NTN1 also induces proliferation of the adjacent periotic mesenchyme, which is required to push together the opposing walls of the otic vesicle to create the fusion plate (Salminen et al., 2000). Similar to NTN1, FGF9 stimulates proliferation of the surrounding otic mesenchyme that is important for fusion plate formation (Pirvola et al., 2004). HMX2 and HMX3 control a later step in fusion plate formation than NTN1. The fusion plate epithelia is able to detach from the underlying basement membrane and come close to each other, whereas the fusion event itself fails in the absence of *Hmx* genes (Hadrys et al., 1998; Wang et al., 2001).

Fusion plate cells are thought to be removed either by migration to the duct rim in the mouse or by apoptosis or transformation into mesenchymal cells in chicken (Martin and Swanson, 1993; Fekete et al., 1997; Kobayashi et al., 2008).

1.3.3.2.3 Continued growth of semicircular ducts

After fusion plate clearance, the growth of the semicircular ducts continues by increasing the diameter of the lumen of semicircular ducts as well as the length of the ducts. Semicircular ducts continue to grow in overall size as the whole inner ear and embryo itself enlarges. NR4A3 (also called NOR1), which encodes a ligand-independent member of the nuclear receptor superfamily of transcription factors, is expressed in the inner walls of the semicircular duct epithelia, and deficient embryos develop abnormally narrow semicircular ducts as a result of reduced proliferation in the epithelium (Ponnio et al., 2002).

1.3.3.3 Regulation of cochlear morphogenesis

The continuous cochleo-saccular structure originates from the ventral region of the otic vesicle. During morphogenesis, the connection that eventually separates the expanding saccule and elongating cochlear duct begins to thin (Figure 4E). The cochlear duct is apparent at E10.75 in mice and at HH23 in chicken (Bissonnette and Fekete, 1996; Cantos et al., 2000).

Several findings suggest that localized programmed cell death at the base of the cochlear duct is important for its outgrowth in mouse and chicken. This cluster of dying cells has not been described in *Xenopus* and zebrafish, which also lack a cochlear structure (Bever and

Fekete, 1999; Cecconi et al., 2004; León et al., 2004). Importantly, lack of programmed cell death in mouse mutants cause a reduction in cell proliferation and in the cochlear duct length (Cecconi et al., 2004). In addition to growth, localized programmed cell death is also involved in the separation of the cochlea and saccule by trimming the epithelium into a thin cochleo-saccular duct, called ductus reuniens (Cecconi et al., 2004).

Over time, the cochlear anlage elongates in its distal region until the mature coiled cochlear duct achieves 1.75 turns in mice, while it remains uncoiled in chicken (Figure 1; Torres et al., 1996; Bissonnette and Fekete, 1996). A number of intrinsic transcription factors are known to control the formation of the cochlear duct and defects in the length and shape of the duct have been described in many gene targeted mouse models (Cantos et al., 2000). *Pax2*-deficient mice show the most severe phenotype with complete agenesis of the cochlea and its associated cochlear ganglion, while the vestibular development occurs relatively normally (Favor et al., 1996; Torres et al., 1996). A milder cochlear phenotype can be seen in mice carrying a hypomorphic allele of *Eya1* (Eya^{bor}), which develop a truncated cochlear duct (Johnson et al., 1999). In humans, allelic defects in *Eya1* also results in cochlear abnormalities often linked to Branchio-oto-renal (BOR) and BO syndromes (Abdelhak et al., 1997; Vincent et al., 1997). Little data is available about the formation of the cochlear coil and mechanisms that regulate the number of coils in different species. OTX1 and OTX2, however, are known to regulate the correct length and coiling of the mouse cochlear duct in a dose-dependent manner (Morsli et al., 1999).

Moreover, extrinsic signals are involved in cochlear development. The correct signaling between the periotic mesenchyme and ventral border of the otic vesicle epithelium is significant for cochlear morphogenesis. Interestingly, POU3F4, also called BRN4, mediated signals from the mesenchyme controls the number of turns of cochlear duct in indirect manner (Phippard et al., 1999; Kiernan et al., 2002). Conditional inactivation of *Tbx1* in the periotic mesenchyme results in failed cochlear duct outgrowth (Xu et al., 2007). *Brn4-/-;Tbx1+/-* double mutant mice develop aberrant spiraling growth of the cochlea resembling human Mondini dysplasia found in patients with Velocardiofacial syndrome/DiGeorge syndrome and X-linked non-syndromic form of deafness (Braunstein et al., 2008).

1.3.4 Sensorineural determination and differentiation

The inner ear hair cells are secondary sensory cells that are innervated by the primary sensory neurons of the vestibulocochlear ganglion, the VIIIth cranial ganglion, which transmit electrical impulses to the central nervous system (CNS; Fritzsch et al., 2002). Both the inner ear sensory cells and ganglionic neurons are derived from a general anteroventromedial portion of the otic cup and vesicle epithelium (Fekete and Wu, 2002; Bell et al., 2008). In mouse and chicken, specification of the presumptive sensory organs and the neurogenic region is controlled by the NOTCH signaling pathway, as well as by FGF signaling and several otic transcription factors (Pirvola et al., 2000; Fekete and Wu, 2002; Abelló and Alsina, 2007).

Fritzsch and Beisel (2004) proposed that the vertebrate hair cells and their innervating neurons evolved from ciliated mechanosensory cells found in insect scolopidal organs. According to this suggestion, both hair cells and neurons would develop from a common multipotent neurosensory progenitor cells. A clonal analysis in both mouse and chicken otic epithelium detected only a few common progenitors for sensory hair cells and neurons, however, and the origin from separate lineages appeared more prevalent (Matei et al., 2005; Satoh and Fekete, 2005).

1.3.4.1 Development of the vestibulocochlear ganglion

The inner ear vestibulocochlear ganglion is formed by bipolar neurons that connect sensory hair cells with neurons in vestibular or auditory brainstem nuclei. Ganglionic cells are derived from two different sources, the epithelial otic placode gives rise to the afferent neurons that innervate the inner ear sensory organs, whereas the neural crest cells give rise to glial cells (Kiernan et al., 2002). The vestibular and cochlear neuroblasts are amongst the first cell types to be specified in inner ear development (Fekete and Wu, 2002).

During the otic placode invagination, a number of epithelial cells known as neuroblasts detach and move into the mesenchymal space at E9.5-10.5 in mice and at HH13-21 in chicken (Adam et al., 1998; Torres and Giráldez, 1998). These precursors of neurons divide and eventually coalesce to form a fused vestibulocochlear ganglion that later separates into vestibular and cochlear ganglion locating closely to the vestibular and cochlear sensory organs (Torres and Giráldez, 1998; Sánchez-Calderón et al., 2007). One major difference between the innervation of vestibular and cochlear sensory organs in the mouse and chicken is the presence of a vestibular sensory organ macula lagena at the distal tip of the chicken cochlear duct. Interestingly, the macula lagena is innervated by the vestibular neurons that are dispersed along the outer edge of the cochlear ganglion rather than aggregated with the other vestibular neurons (Fekete and Campero, 2007).

Subsequent to proliferation, sensory neurons precursors exit the cell cycle, become post-mitotic, and extend their axons to make a synaptic connection with one of the sensory organs and with either vestibular or auditory brainstem nuclei (Fekete and Wu, 2002; Fritzsch et al., 2002). The mechanisms guiding the delamination of neuroblasts and afterward the axon pathfinding back to the sensory epithelium are largely unknown. Fritzsch et al. (2002) proposed that delaminating neuroblasts may carry positional information to direct axons back to the sensory organ. The survival and differentiation of ganglionic neurons depends on neurotrophic proteins. The neurons associated with cristae require brain-derived neurotrophic factor (BDNF), whereas cochlear neurons depend on glial cell line-derived neurotrophic factor (GDNF), as well as neurotrophin 3 (NTF3) and BDNF (Ylikoski et al., 1998; Fritzsch et al., 2004; Roehm and Hansen, 2005).

1.3.4.1.1 Regulation of neuronal fate by proneural genes and NOTCH signaling

The vestibular and cochlear neuroblasts arise from separate developmental compartments of the otic epithelium (Fekete and Wu, 2002). This has been confirmed by a fate map of the chicken otic placode demonstrating that the anterolateral region of the otic epithelium generates vestibular neurons, while the posteromedial region gives rise to the cochlear neurons. Additionally, there is a temporal difference between the two classes of neurons as precursors of vestibular neurons start to delaminate earlier than cochlear neuroblasts (Bell et al., 2008).

The initiation of neuronal lineages, promotion of delamination of neuronal precursors from otic epithelium, and neuronal differentiation are controlled by proneural genes, encoded by transcription factors of the basic Helix-Loop-Helix (bHLH) family (Bertrand et al., 2002). The early steps in otic neurogenesis are regulated by two essential bHLH genes, *Neurogenin 1* (*Neurog1*) and *Neurod1*. In the mouse, NEUROG1 is required for the generation of vestibular and cochlear neuronal precursors (Ma et al., 1998). The loss of sensory neuron formation in *Neurog1* mutants is mediated through a phenotypic switch of neurosensory precursors from sensory neuron to hair cell fate (Matei et al., 2005). Unlike NEUROG1, NEUROD1 is involved in neuroblast delamination rather than neural cell fate specification (Kim et al., 2001). In chicken, the expression of *Neurog1* and *Neurod1* are positively regulated by FGF signaling that is essential for neuronal fate in otic epithelium (Alsina et al., 2004). During mouse and chicken otic neurogenesis, members of the FGF family are important in processes

of neuroblast delamination, proliferation, survival, apoptosis, and differentiation in the vestibulocochlear ganglion. Accordingly, the expression of *Fgf10* in the otic cup and vesicle epithelium coincides with the neurosensory competent region in both mouse and chicken embryos (Pirvola et al., 2000; Alsina et al., 2004).

The neuronal development requires an interplay between proneural genes and NOTCH signaling (Bertrand et al., 2002). NOTCH signaling is essential in mediating the decision between neural versus otic epithelial cell fate during inner ear development. Hence, the activated NOTCH signaling pathway is involved in the process of lateral inhibition, which prevents neighboring cells from acquiring the same fate. Within the otic placode and vesicle, *Notch1* is widely expressed, whereas one of its ligands, *Delta1-like* (*Dll1*), is expressed in isolated cells in anteroventral region of the otic epithelium, presumably the neurogenic region (Ma et al., 1998; Morrison et al., 1999). Neuroblasts that express *Dll1* inhibit their adjacent cells from obtaining the same fate and thus controlling the number of developing neurons (Fekete, 2004). This is supported by the inhibited NOTCH signaling pathway in *mind bomb* zebrafish mutants, which generates an excessive number of ganglion neurons from the otic epithelium (Haddon et al., 1998).

1.3.4.2 Sensory determination

A common prosensory region in the ventromedial wall of the otic vesicle, which overlaps with the region generating neurons, gives rise to all the different sensory organs in the mouse and chicken inner ears (Bryant et al., 2002). Some sensory neurons and hair cells may share a common progenitor as well (Matei et al., 2005; Satoh and Fekete, 2005). Hence, the loss of NEUROG1 affects both sensory neuron and hair cell formation in the mouse inner ear (Ma et al., 1998; 2000). In fact, it has a role in determining the fate of neurosensory precursor cells becoming hair cell and neurons (Matei et al., 2005).

As with ganglionic neurons, the formation of hair cells critically depends on NOTCH signaling and proneural genes. NOTCH signaling pathway members are among the earliest genes expressed in the sensory organ anlage in the mouse and chicken. The expression of the NOTCH ligand *Jagged 1* (*Jag1*) in mice (named *Serrate 1* (*Ser1*) in chicken), and the NOTCH pathway modulator, *Lunatic fringe* (*Lfng*) have been detected in the presumptive sensory anlage (Adam et al., 1998; Morsli et al., 1998; Morrison et al., 1999; Cole et al., 2000). Daudet and colleagues (2007) have shown that SER1 maintains NOTCH activation in chicken and thereby preserves and possibly extends the prosensory state in otic epithelium. In mice, conditional inactivation of *Jag1* also leads to a severe reduction of the sensory epithelium, suggesting that NOTCH signaling mediated via the JAG1 ligand, is essential for the induction and maintenance of the prosensory fate (Brooker et al., 2006; Kiernan et al., 2006). Recent data, however, contradict these findings and suggest instead that NOTCH/JAG1 signaling is not actually necessary for prosensory induction, but rather to control cell survival in the developing mouse cochlea (Basch et al., 2011). From the proneural genes, *Atoh1* (also called *Math1*) is required for the genesis of vestibular and cochlear hair cells (Bermingham et al., 1999). However, ATOH1 appears to act at relatively late stage and it does not really have a true proneural function in inner ear (Müller and Littlewood-Evans, 2001).

In addition to the proneural function and NOTCH signaling, a member of the SOX transcription factor family, SOX2, has a crucial role in prosensory region determination in mice. SOX2 is one of the first transcription factors expressed in the prosensory domain of the otic vesicle (Mak et al., 2009) and its inactivation leads to a failure in the establishment of the prosensory region (Dabdoub et al., 2008). Mouse mutants with reduced expression of *Sox2* show disturbed arrangement and reduced numbers of hair cells and supporting cells (Kiernan et al., 2005a). Gain-of-function experiments in mice suggest a signaling cascade in the

developing sensory epithelium in which JAG1-mediated NOTCH signaling activates *Sox2* expression, which in turn induces expression of *Prox1*, a homeodomain transcription factor regulating cellular differentiation in the inner ear sensory epithelia (Dabdoub et al., 2008; Kirjavainen et al., 2008).

1.3.4.3 Specification and development of distinct sensory organs

One interesting but little understood morphogenetic event in inner ear development is the subdivision of the neurosensory competent domain into six distinct sensory regions of the otic epithelium. In addition, the molecular mechanisms that govern the specification of a particular sensory organ, are largely unknown.

The approximate timing of a particular sensory organ specification can be estimated according to the expression pattern of certain genes, that specifically mark the sensory organ primordia. For example, *Bmp4* expression is detected in the presumptive cristae at E9.0-10.5, and *Lfng* marks the primordia of the two maculae and the organ of Corti around the same time (Morsli et al., 1998).

Expression analyses have shown that the prosensory domains of the otic epithelium progressively divide into distinct sensory structures during development. The *Bmp4*-positive region becomes first divided into two, forming an anterior and a ventral domain. Then, the anterior region splits into two again, forming the superior and lateral cristae, while the ventral domain gives rise to the posterior crista. The *Lfng*-positive region becomes divided into a dorsal and ventral region. The dorsal region is destined to become the utricular macula, and the ventral region encompasses the future saccular macula and the cochlear sensory region which remain together for a longer periode (Morsli et al., 1998). These results suggested that distinct sensory organs share a common origin at early stages in mouse (Morsli et al., 1998). In contrast to rodents, all sensory organs of the chicken inner ear are thought to arise independently from each other (Wu and Oh, 1996).

NOTCH signaling together with WNT and BMP4 are the main regulators to control the proper size, organization, and character of inner ear sensory organs. In the inner ear, WNT signaling is one of the pathways that govern sensory organ type and development. Interestingly, ectopic activation of the canonical WNT signaling pathway in the chicken otic vesicle before sensory organ specification leads to the formation of ectopic sensory patches of hair cells in certain nonsensory regions, and changes the cochlear sensory organ character into a vestibular one (Stevens et al., 2003). Nevertheless, the role of the endogenous WNT pathway in sensory organ specification has not been proven. Similar to the WNT signaling, a consitutively active form of NOTCH induces the formation of ectopic sensory patches in certain regions of the inner ear. Consequently, NOTCH signaling may be important for the induction of sensory organs as well as for their maintenance (Daudet and Lewis, 2005). Li et al. (2005) demonstrated in *in vitro* test that the loss of BMP4 in cultured otic vesicles makes the epithelial cells unable to commit to a sensory cell fate. In the chicken, blocking of BMP activity with an exogenous BMP inhibitor, NOGGIN protein yielded inner ears with malformed sensory epithelia suggesting that BMP signaling is required for the differentiation and possibly for the identity and position of otic sensory organs (Chang et al., 1999).

1.3.4.4 Cell fate determination in sensory organs

Clonal analysis in chicken has revealed that hair cells and supporting cells arise from the same precursor cell (Fekete et al., 1998). Determination of becoming a hair cell or supporting cell is made through lateral inhibition. Thus, NOTCH signaling has two distinct functions in inner ear development, an early inductive role in prosensory fate determination and later an inhibitory role inside the prosensory epithelium (Brooker et al., 2006). The lateral inhibition model suggests that cells in the presumptive sensory epithelium are equipotential about their

fate and express both *Notch1* and its ligand(s). For unknown reasons, at a certain time point some cells in the epithelium upregulate ligand expression and thus activate NOTCH signaling in the surrounding cells. Eventually, high levels of NOTCH activation leads to hair cell fate inhibition and the NOTCH-expressing cells differentiate into supporting cells (Kiernan et al., 2002).

In the process of lateral inhibition, the *Hes*-genes, *Hes1* and *Hes5*, act as downstream targets of NOTCH signaling. Accordingly, deletions of the *Hes*-genes produce supernumerary hair cells in the organ of Corti (Zine et al., 2001). The lateral inhibition model is supported by the expression of genes encoding NOTCH ligands, DLL1 and JAG2 in hair cells and *Notch1* in supporting cells (Lanford et al., 1999; Morrison et al., 1999). Furthermore, *Jag2* mouse mutants produce extra rows of inner and outer hair cells in the cochlea (Lanford et al., 1999) and tissue-specific inactivation of *Dll1* results in overproduction of cochlear hair cells at the expense of supporting cells (Brooker et al., 2006), indicating defects in lateral inhibition. A more severe cochlear phenotype with supernumerary and extremely disorganized hair cells has been observed in mouse mutants that lack both copies of *Jag2* and carry either a single null allele of *Dll1* ($Dll1^{+/-}/Jag2^{-/-}$) or the null allele combined with a *Dll1* hypomorphic allele ($Dll1^{hyp/-}/Jag2^{-/-}$) (Kiernan et al., 2005b).

Unlike the other NOTCH ligands, *Jag1* is expressed in the supporting cells of the organ of Corti together with *Notch1* (Morrison et al., 1999). Conditional inactivation of *Jag1* leads to the overproduction of inner hair cells and lack of outer hair cells and associated supporting cells (Brooker et al., 2006). This curious cochlear phenotype has been explained as the failure of prosensory induction in *Jag1*-deficient cochlea leading to the absence of outer hair cells. In addition, the observed loss of a cyclin-dependent kinase inhibitor 1B (CDKN1B; also called $p27^{Kip1}$) in *Jag1* mutants may reflect increased proliferation that would eventually lead to overproduction of inner hair cells.

1.3.4.5 Differentiation of hair cells

Hair and supporting cells undergo their terminal mitosis between E11.5 and birth in mice. In the vestibular sensory epithelia, there is a center to periphery gradient of terminal mitosis and differentiation appears to follow the same spatial pattern as that observed for the final mitosis (Kiernan et al., 2002). In cochlea, the gradient of terminal mitosis starts in the apical part and continues to the base close to the saccule. Immediately after cell cycle exit, differentiation of the cochlear epithelium occurs in the opposite direction to that seen with final mitosis: from base to apical. In addition to the longitudinal gradient, a horizontal gradient of differentiation also exists, as the inner hair cells begin to differentiate before the outer hair cells. Terminal mitosis and cell cycle exit are controlled by cell cycle inhibitors, for instance Retinoblastoma (Rb) proteins and their upstream regulators CDKN1B, CDKN1A (also called $p21^{Cip1}$), and CDKN2D (also called $p19^{INK4d}$) (Liu and Zuo, 2008).

The differentiation of both vestibular and cochlear hair cells depends specifically on a proneural bHLH transcription factor ATOH1. Mouse mutants deficient for *Atoh1* completely lack the sensory hair cells (Bermingham et al., 1999). Furthermore, overexpression of *Atoh1* induces hair cell generation in neonatal rat cochlear explants as well as guinea pig inner ears *in vivo* (Zheng and Gao, 2000; Kawamoto et al., 2003). On the other hand, hair cell generation is inhibited by the HES family bHLH proteins that negatively regulate *Atoh1* (Kelley, 2006). Lastly, the terminal differentiation of hair cells is dependent on a POU domain factor, POU4F3 (Erkman et al., 1996; Xiang et al., 1998).

1.4 Sensorineural hearing disorders and regeneration of sensory hair cells

Hearing loss is the most common sensory disorder in humans. Approximately 50% of people over 65 suffer from some degree of hearing loss (Pauley et al., 2008). More than 60% of all incidents are caused by genetic factors (Piatto et al., 2005). The leading source of deafness in humans is sensorineural hearing loss caused by damaged inner ear cochlear sensory hair cells that in turn results in a degeneration of cochlear ganglion neurons (Li et al., 2003a).

Most non-mammalian vertebrates can either regenerate new hair cells in response to damage or undergo continuous replacement of hair cells during their life span (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Stone and Rubel, 2000; Bermingham-McDonogh and Rubel, 2003; Stone and Cotanche, 2007). In mammals, however, new cochlear hair cells are induced through direct transdifferention from supporting cells only during a limited period of embryonic development, and therefore, loss of hair cells causes permanent hearing disorders (Bermingham-McDonogh and Rubel, 2003). Some spontaneous regeneration of hair cells does occur in mammalian vestibular epithelia (Forge et al., 1998).

Understanding the molecular pathways and mechanisms controlling regeneration in the mouse sensory epithelium may provide a valuable key to designing new therapies for human deafness.

1.4.1 Molecular background of sensorineural deafness in humans and domestic animals

Approximately 70% of inherited deafness cases are non-syndromic, and the remaining 30% are syndromic, associated with other clinical abnormalities. More than 400 genetic syndromes are associated with hearing impairment in humans, while 46 genes have been identified as involved in non-syndromic hearing impairment (Toriello et al., 2004; Hilgert et al., 2009). Mutations in these 46 genes either affect sensory hair cells directly, or cause alterations in the surrounding cells that in turn lead to the impairment of hair cell functions. Below are some examples of deafness genes.

Maintaining ion homeostasis within the cochlear duct, and especially the high K^+ concentration in the endolymph, is extremely important for signal transduction involved in the hearing process. In the cochlear duct, stria vascularis secretes the endolymph and generates the largest transepithelial voltage in the whole organism, the endocochlear potential. In principle this is the K^+ equilibrium potential between endolymph and perilymph, and is generated by the K^+ channel KCNJ10 located in the special cells of stria vascularis (Marcus et al., 2002). Consequently, mutations in several genes encoding K^+ channel proteins cause severe hearing impairment (Jentsch, 2000; Wangemann et al., 2004).

Gap junctions are channels allowing transport of ions and small metabolites between neighboring cells and consist of two homo- or heterohexameric hemichannels of connexin transmembrane proteins. In humans and mice, around 20 connexin encoding genes have been identified. Several *connexin* genes are expressed in the mouse cochlea, two of which, *connexin26* (*Gjb2*) and *connexin30* (*Gjb6*), are coexpressed in supporting cells and fibrocytes and are considered critical for recyling endolymphatic K^+ ions (Willecke et al., 2002; Forge et al., 2003; Wangemann, 2002; Martínez et al., 2009). Inactivation of murine *Gjb2* and *Gjb6* cause deafness (Kudo et al., 2003; Teubner et al., 2003). Notably, mutations in *Gjb2* and *Gjb6* are the most frequent genetic causes of nonsyndromic deafness in humans (del Castillo et al., 2002; Petersen and Willems, 2006).

Alterations in several transcription factor-encoding genes also result in syndromic deafness. For example, mutations in a paired box gene *Pax3* cause Waardenburg syndrome characterized by sensorineural hearing loss associated with skin and hair pigmentary anomalies (Karaman and Aliagaoglu, 2006). The main clinical features of BOR syndrome

include branchial arch defects, hearing loss, and renal anomalies. Defects in members of EYA and SIX transcription factor families have been identified in BOR syndrome (Hoskins et al., 2007; Sanggaard et al., 2007). Mutations in the gene encoding for transcription factor GATA3 also cause HDR syndrome characterized by the hypoparathyroidism, deafness, and renal dysplasia (Nesbit et al., 2004).

Pendred syndrome is the most frequent form of syndromic hearing loss and is associated with mutations in the *Slc26a4* gene encoding a protein called pendrin. Pendred syndrome is characterized by severe sensorineural hearing loss, enlarged membranous labyrinth, and thyroid abnormalities (Everett et al., 2001). The transmembrane protein pendrin functions as an anion transporter and thus maintains the ionic balance within the endolymph (Everett et al., 1999). Not surprisingly, *Slc26a4* is expressed in areas known to play a role in endolymph resorption, such as the endolymphatic duct and sac epithelium, and special cells in the cochlear duct (Everett et al., 1999).

In domestic animals, deafness has mainly been observed in dogs and cats. Congenital deafness in dogs has been documented in more than 80 breeds (Strain, 2004). Most studies focused on the Dalmatian, with high deafness frequencies (20%) in the United States of America and United Kingdom (Rak and Distl, 2005). The deafness in most dogs and cats is associated with skin pigmentation genes and often white hair and blue eyes correlate hearing abnormalities. The pigment-associated deafness is a result of the loss of melanocytes. These melanin-producing cells determine the color of the skin or hair and are also present in the stria vascularis of the cochlea where they produce endolymph and its positive (+80mV) electrical potential. The endocochlear potential essential for the transduction of sound by hair cells (Cvejic et al., 2009; Hibino et al., 2010).

1.4.2 Induction of hair cell regeneration – restoring hearing loss?

Acoustic trauma can damage mammalian hair cells, treatment with certain antibiotics, infections, or parts of the aging process cause permanent hearing deficit due to limited regenerating capacity of hair cells. Natural regeneration of the cochlear sensory epithelia does happen in fish, amphibians, and birds. In adult birds, new cochlear hair cells can regenerate after damage either by proliferation and differentiation of neighboring nonsensory supporting cells or by direct transdifferentiation into sensory hair cells without cell division (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Roberson et al., 2004; White et al., 2006; Stone and Cotanche, 2007). Studies in both lower vertebrates and mammals have uncovered genes and pathways important in hair cell development and have suggested ways to achieve hair cell regeneration.

Unlike birds, the precursors of hair cells and supporting cells in the mammalian cochlea undergo terminal mitosis during embryogenesis and are unable to re-enter the cell cycle after damage. Therefore, the re-entry of the post-mitotic supporting cells in the cell cycle appears to be key in hearing loss therapies. The cell cycle arrest in sensory progenitor cells is mainly regulated by CDKN1B and RB1. CDKN1B is later involved in the cell cycle arrest of cochlear supporting cells, whereas RB1 plays an essential role in the maintenance of the quiescent state of cochlear hair cells and supporting cells (Chen and Segil, 1999; Löwenheim et al., 1999; Mantela et al., 2005; Sage et al., 2005). Indeed, targeted mutagenesis of *Cdkn1b* leads to development of supernumerary hair cells and supporting cells, and causes ongoing cell proliferation in the adult mouse organ of Corti (Chen and Segil, 1999; Löwenheim et al., 1999). Furthermore, the conditional tissue-specific deletion of *Rb1* in mice results in increased number of sensory cell progenitors that develop into hair cells and supporting cells (Sage et al., 2006). *Rb1*-deficient mice show supernumerary cochlear hair cells produced via hair cell proliferation. Hair cells in these animals, however, display increased levels of programmed cell death and alterations in the stereociliary bundle morphology (Mantela et al.,

2005). In addition, Sulg et al. (2010) proved that forced cell cycle re-entry in post-mitotic terminally differentiated cochlear outer hair cells leads to rapid cell death due to p53- induced DNA damage. Moreover, post-mitotic supporting cells isolated from adult mouse cochlea have the ability to downregulate *Cdkn1b* expression, divide, and transdifferentiate into hair cells when cultivated together with embryonic periotic mesenchyme (White et al., 2006). Understanding the negative cell cycle regulation of hair cells and supporting cells is one of the most important aspects for finding therapies for deafness caused by hair cell damage.

Another promising factor for deafness therapy may be the proneural transcription factor ATOH1, which is essential for the generation of hair cells. Overexpression of *Atoh1* in cultures of neonatal rat cochleas results in the production of ectopic hair cells (Zheng and Gao, 2000). Furthermore, delivery of an adenoviral vector encoding ATOH1 into nonsensory cells of a mature deaf guinea pig cochlea leads to regeneration of hair cells and improved hearing (Izumikawa et al., 2005).

Stem cells could also be used to replace lost hair cells within the mammalian cochlea. In the adult mouse inner ear, the sensory epithelium of the utricle contains stem cells that have the capacity of self-renewal, and *in vitro* they form spheres expressing otic marker genes. These inner ear stem cells can differentiate into hair cell-like cells (Li et al., 2003a). Moreover, embryonic stem cells and stem cells from other tissues (neural tissue, hematopoietic system) also show capacity to differentiate into hair cells and cochlear neurons, depending on the experimental setting (Li et al., 2003b; Hu et al., 2005; Matsuoka et al., 2007). Many findings illustrate the promise of stem cell therapy in the treatment of hair cell loss, however, in my opinion further investigation in animal models will be necessary to achieve success.

1.5 Development and structure of the midbrain

Neural development is one of the earliest to begin and last to be completed after birth. Gastrulation (E7-8 in mouse) is a crucial time in the development of multicellular organisms as three germ layers (ectoderm, mesoderm, and endoderm) are established during this stage. Different organs are derived from these three embryonic layers, and the ectoderm gives rise to the nervous system and epidermis. The fate decision to become neural or epidermal tissue is regulated by BMP4, which functions as an epidermal inducer and neural inhibitor (Wilson and Hemmati-Brivanlou, 1995).

The development of the CNS starts shortly after gastrulation when cuboidal epithelial cells become columnar and form a thickening of ectodermal cells called a neural plate. Thereafter, the edges of the neural plate move upward and towards the midline of the embryo. Eventually, the neural folds adhere to each other and form a closed cylinder-like neural tube.

The early mammalian neural tube is a fairly straight structure. The anterior portion of the neural tube, however, undergoes drastic changes even before the posterior portion of the tube has formed. The anterior neural tube expands into three primary vesicles: forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). In subsequent stages, prosencephalon and rhombencephalon divide into two and consequently produce five secondary vesicles: telecephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. These vesicles give rise to different structures of the brain based upon a combination of transcription factors expressed in the vesicles, and signaling molecules from surrounding tissues. The mesencephalon ultimately becomes subdivided along the dorsoventral axis into the tectum (dorsal midbrain) and the tegmentum (ventral midbrain) (Figure 5; Echevarría et al., 2003).

The midbrain is mainly composed of dense tracts of neurons passing from the forebrain to the spinal cord, but it also contains the nuclei (ganglion) of one cranial nerve: the

oculomotor nerve (cranial nerve III; Figure 5). The differentiation of the dorsal midbrain (tectum) results in the formation of four swellings, which form structures involved in visual and auditory reflexes. The rostral two swellings form the superior colliculi, and the caudal two swellings form the inferior colliculi. The superior colliculus is a center of visual reflex that helps to orient the head and eyes to all types of sensory stimuli. The inferior colliculus is an auditory structure that is involved in analyzing the spatial location of sound (Greenstein and Greenstein, 2000). Compared to the dorsal midbrain, the ventral midbrain does not have a clear distinction between structures. Nevertheless, tegmentum includes the ventral tegmental area, periaqueductal gray matter, reticular formation, red nucleus, and substantia nigra. These regions are involved in pain processing, fear, anxiety, respiratory control, regulation of sleep and wakefulness, as well as coordination of eye movements, motor control and emotion processing (Behbehani, 1995; Monti and Jantos, 2008).

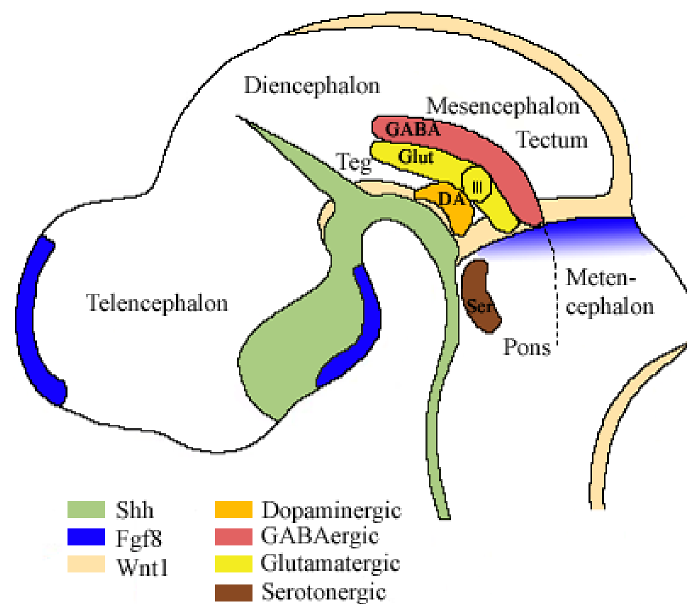

Figure 5. Schematic drawing of the rostral parts of the mouse neural tube at E11.0 with neuronal cell populations and expression domains of signaling molecules in different colors. The expression of genes for secreted factors are shown at the mid-hindbrain boundary (*Fgf8*, *Wnt1*), in the anterior neural ridge, and ventral diencephalon (*Fgf8*), in the dorsal midline of the midbrain, mesencephalic flexure, and dorsal midline of the posterior hindbrain (*Wnt1*), and within the caudal forebrain, midbrain, and hindbrain (*Shh*). Neuronal populations (GABAergic, glutamatergic, dopaminergic, and serotonergic) are indicated. Cranial motor neurons in the midbrain form the oculomotor nerve (cranial nerve III). Teg, tegmentum.

1.5.1 Anteroposterior patterning of the midbrain

Segmentation of the neural tube into morphogenetic units is achieved by local signaling centers with polarizing and inductive properties. Signaling centers develop within the broadly regionalized neuroectoderm in genetically defined positions. The primary organizer (node) establishes early polarity of the embryos, and the secondary organizers (anterior neural ridge, isthmic organizer (IsO)) further refine the patterns laid down by the primary organizers (Echevarría et al., 2003).

IsO is located at the border between the posterior midbrain and anterior hindbrain and regulates the anteroposterior patterning of the midbrain (Nakamura et al., 2005). At the end of gastrulation, the homeobox-domain-containing transcription factors OTX2 and GBX2 are

expressed in the mouse embryo in a largely complementary manner in the anterior and posterior neuroectoderm (Wassarman et al., 1997; Simeone et al., 2002). The position of IsO is defined and maintained by the meeting point of the regions that express *Otx2* and *Gbx2* (Wurst and Bally-Cuif, 2001).

The IsO is essential for the differentiation of both mouse and chicken midbrain through long-range action of secreted signals (Martínez et al, 1999; Martínez, 2001). Especially the two signaling molecules, WNT1 and FGF8, secreted by IsO control the patterning of cells within the midbrain (Mason et al., 2000; McMahon et al., 1992). FGF8 acts through the homeodomain proteins, Engrailed 1 (EN1) and Engrailed 2 (EN2), GBX2, OTX2, and PAX6 to arrange the anteroposterior patterning of cells (Puelles et al., 2004). Loss-of-function analyses demonstrated that FGF8 and WNT1 are required for the formation of the entire midbrain and anterior hindbrain region (McMahon et al., 1992; Chi et al., 2003). Interestingly, the isthmic tissue grafts are able to transform caudal forebrain into an ectopic midbrain in chicken (Crossley et al., 1996). This ectopic midbrain forms under the influence of signals from a new isthmus-like organizing center induced in the forebrain. The major player here is FGF8 that induces the nearby cells to express *En2*, *Fgf8*, and *Wnt1*, thereby inducing an ectopic IsO. Thus, FGF8 protein seems to have similar midbrain inducing and polarizing effects as isthmic tissue (Crossley et al., 1996).

1.5.2 Dorsoventral patterning of the midbrain

The dorsoventral patterning in CNS is best characterized in the spinal cord, where the polarity is generated by opposing sets of signals originating from the notochord and the dorsal ectoderm of the embryo. The dorsoventral pattern of the spinal cord depends on the relative amounts of ventralizing (SHH) and dorsalizing (BMP) factors. Based on the graded concentration of the two factors, distinct classes of neurons are formed in the spinal cord: sensory neurons differentiate in the dorsal region, motor neurons in the ventral region, and interneurons between them.

Nakatani et al. (2007) identified seven dorsoventral domains in the midbrain neuroepithelium. These progenitor domains express distinct combinations of transcription factors and give rise to different populations of neurons in the midbrain. Similar to the spinal cord, SHH induces ventral gene expression and represses dorsal genes (Agarwala et al., 2001; Bayly et al., 2007).

1.5.3 Neuronal cell types in midbrain

Neurons in the midbrain serve many essential functions. The midbrain contains the largest group of dopamine-producing (dopaminergic) neurons. The dopaminergic neurons in the substantia nigra and the ventral tegmental area transmit signals concerned with motor function to parts of the forebrain, and regulate the mood and behavioral state of an individual. Another cell type, serotonergic neurons, are mainly found in the dorsal raphe nuclei, the most anterior part of the raphe nuclei located in the midbrain. The raphe nuclei have an enormous impact on behavior regulating wake and sleep cycles, affective behavior, food intake, and thermoregulation (Monti and Jantos, 2008).

The largest groups of cholinergic neurons in the brain are found in the midbrain and the basal forebrain. The neurons in the tegmental nuclei of the midbrain provide cholinergic innervation to the brainstem and the thalamus that is critical for inducing a state of cortical arousal, both during wakefulness and dreaming (Kayama and Koyama, 2003). Additionally, GABAergic neurons in the midbrain control several aspects of behavior, including voluntary and involuntary movements, mood, motivation, and addiction (Korotkova et al., 2004). The function of a small group of putative glutamatergic neurons within the ventral tegmental area is still unclear (Geisler and Wise, 2008).

1.5.3.1 Determination and specification of glutamatergic and GABAergic neurons

Two major neuron types in the brain are glutamatergic (projection neurons) and GABAergic (interneurons). The projection neurons are mostly excitatory glutamatergic, whereas the interneurons are inhibitory GABAergic ones.

The acquisition of the GABAergic or glutamatergic phenotype is controlled by distinct pathways in different brain areas. For example, a dynamic choice between GABA- and glutamatergic fate occurs in closely related cell linages during dorsal spinal cord development. In this region, the homeodomain and bHLH transcription factors play essential roles in the specification of identity of the progenitors and derived neurons (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Mizuguchi et al., 2006). Additionally, *Ptf1a*, *Tlx1*, and *Tlx3* function as post-mitotic selector genes that instruct the neurotransmitter fate selection (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005). In the telencephalon, however, the proneural bHLH factors ASCL1 (MASH1) and NEUROG2 are selectively expressed in progenitors for GABAergic and glutamatergic neurons and are involved in the determination of the neurotransmitter phenotype (Fode et al., 2000; Parras et al., 2002). Nakatani et al. (2007) identified a novel bHLH-Orange (bHLH-O) family member, *Helt*, that determines the GABAergic over glutamatergic neuronal fate in the developing midbrain. Furthermore, *Neurog1* and *Neurog2*, which show activity for promoting glutamatergic neuron differentiation, are downstream target genes of HELT.

Specification of glutamatergic and GABAergic phenotypes is not controlled by an universal transcriptional program, but rather by region-specific sets of transcription factors. For example, the *Dlx* class homeobox genes, which function in controlling GABAergic differentiation in the forebrain, are not expressed in the hindbrain or the spinal cord (Marín and Rubenstein, 2003). In addition, genes encoding proteins responsible for vesicular glutamate transport, *Slc17a7* (*VGLUT1*) and *Slc17a6* (*VGLUT2*), are expressed in complementary territories in the vertebrate brain (Kaneko and Fujiyama, 2002; Fremeau et al., 2004).

1.6 GATA transcription factors in development

A central feature of development is to achieve a high variety of cell types and tissues from multipotent stem and progenior cells. The specification of cell fates occurs through the precise combined application of transcription factors. The GATA transcription factor family has numerous and unique roles during development. Notably, GATA factors are involved in cell fate specification, cell proliferation, differentiation, and cellular movements (Patient and McGhee, 2002).

Members of the GATA family have been described across eukaryotes: four in *Drosophila*, six in vertebrates, and eleven in *C. elegans*. GATA factors share some common characteristics. Members of GATA family bind the consensus DNA sequence (A/T) GATA (A/G) (Ko and Engel, 1993; Merika and Orkin, 1993), which has given the name GATA for this family. GATA factors have a special protein structure, a zinc finger (ZF) motif, as a DNA binding domain (Patient and McGhee, 2002; Cantor and Orkin, 2005). GATA proteins contain two highly conserved ZF domains (Figure 6). The carboxy-terminal ZF (C-ZF) and the following basic region is responsible for binding to GATA sequences in the target genes, whereas the amino-terminal ZF (N-ZF) can modulate the binding specificity via interacting with transcriptional coregulators. GATA proteins can interact with diverse partners, for example Friend of GATA (FOG) family members FOG1 and FOG2, LIM-family cofactor NLI and LIM-only transcription factor LMO2 (Cantor and Orkin, 2002; Ferreira et al., 2005). This interaction can either enhance or repress GATA-mediated transactivation depending on

the cell and promoter context. GATA factors can also induce changes in the chromatin structure and modulate transcriptional competence (Patient and McGhee, 2002; Grass et al., 2003; Cantor and Orkin, 2005; Shoemaker et al., 2006).

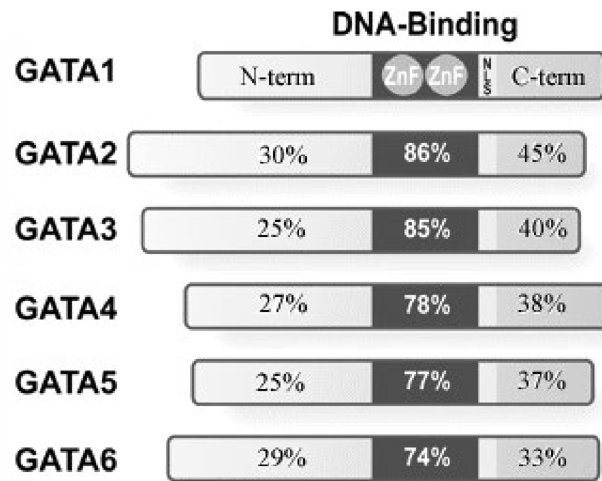

Figure 6. Structure and homology of mouse GATA proteins. GATA factors share a highly similar zinc finger (ZF)-domain. Less similar transactivation domains are located in the N-terminal (N-term) and C-terminal (C-term) regions. The homology in N-terminal, C-terminal and ZF regions between mouse GATA1 and the other GATA factors is presented as % in each domain. NLS, nuclear localization sequence. Modified from Viger et al. (2008).

The highly conserved DNA-binding domain shared by GATA family members shows an apparent redundancy in DNA binding properties to target GATA sequence (Figure 6; Ko and Engel, 1993; Merika and Orkin, 1993). These *in vitro* properties are, however, often in contrast with their nonredundant functions *in vivo* (Tsai et al., 1994; Pandolfi et al., 1995) that could be partially explained by the different transcriptional coregulators interacting with GATA proteins. The expression profiles of GATA factors are also independently restricted, suggesting that they may activate tissue-specific genes and thereby define tissue-specificity.

In vertebrates, the six GATA factors can be divided into two subgroups according to their amino acid sequence and main functions in certain tissues. GATA1, GATA2, and GATA3 are linked to the specification of hematopoietic cell fates, while GATA4, GATA5, and GATA6 play critical roles in the specifation of endodermal tissues, including heart and lung. Moreover, these GATA factors are also involved in various other tissues (Patient and McGhee, 2002; Burch, 2005; Cantor and Orkin, 2005).

The functions and molecular pathways related to GATA2 and GATA3 will be discussed more thoroughly with regard to my particular interest.

1.6.1 GATA2 and GATA3 in embryonic development

Gata2 and *Gata3* are expressed in a highly restricted spatio-temporal manner in a variety of tissues during embryogenesis. Describing the functions of GATA2 and GATA3 in various organs has been complicated, however, because of the early lethality of their knockout mutants. Notably, *Gata2*-deficient mouse embryos die due to anemia at E10.5 (Tsai et al., 1994), and the loss of *Gata3* causes defects in several organ systems leading to death about the same stage, E10.5-11.5 (Pandolfi et al., 1995).

The best-characterized roles and molecular pathways of GATA2, GATA3, and GATA1 have been described during the development of blood cells, which have clarified how the transcription factors with similar specificities have unique functions. GATA2 is crucial for

maintenance and proliferation of multipotent hematopoietic progenitor cells (Tsai and Orkin, 1997). During the differentiation of the erythroid cells, *Gata2* expression is downregulated (Tsai et al., 1994). The *Gata2* repression has been of particular interest, since the enforced *Gata2* expression in hematopoietic progenitor cells alters the development of blood cells (Persons et al., 1999; Kitajima et al., 2002). Basically, the expression of *Gata2* is activated by BMP4-signaling in hematopoietic progenitors where GATA2 maintains its own expression (Maeno et al., 1996). The positive autoregulation of *Gata2* involves a distant upstream region, to which GATA2 binds. Since *Gata2* is expressed in hematopoietic progenitor cells earlier than *Gata1*, it might induce *Gata1* transcription before autoregulation. The accumulation of GATA1 leads to the interaction with the cofactor FOG1 and displacement of GATA2 from the *Gata2* upstream region. Thus, GATA1 disrupts positive autoregulation of *Gata2* and establishes a broad domain of repressive chromatin structure (Grass et al., 2003).

GATA2 functions have been extensively studied in the hematopoietic system, but *Gata2* has also been implicated in regulating urogenital development. A yeast artificial chromosome, which comprises a partial genomic *Gata2* fragment, rescues *Gata2* knockout mice from embryonic lethality, but the transgenic animals have deformed kidneys and urethras that lead to a blockade in urine excretion and finally to perinatal death (Zhou et al., 1998). GATA2 is also essential for the differentiation of white and brown adipocytes. *Gata2* is expressed in adipocyte precursors, and is downregulated during the differentiation of adipocytes (Tsai et al., 2005; Okitsu et al., 2007). In pituitary glands, GATA2 appears to be dispensable for gonadotrope and thyrotrope cell fate and maintenance, but important for optimal function (Charles et al., 2006).

In contrast to GATA2, GATA3 is required for the differentiation of the T helper type 2 cells (Th2). The accumulation of GATA3 eventually leads to the direct or indirect regulation of its own transcription (Ouyang et al., 2000; Ranganath and Murphy, 2001). The autoactivation of GATA3 may have a stabilizing influence in the commitment of Th2 cell fate. Thereafter, GATA3 activates the Th2 cell specific cytokine cluster required for Th1 cell specification (Zheng and Flavell, 1997; Ferber et al., 1999; Shoemaker et al., 2006).

In addition to T-cell development, essential roles of GATA3 have been established in multiple other organs. In kidney development GATA3 is required for the morphogenesis and guidance of the Wolffian duct, and *Gata3*-deficient mutant mouse embryos lack the metanefros (Grote et al., 2006). In skin development, it is involved in the cell lineage determination of the multipotent progenitor cells (Kaufman et al., 2003). GATA3 is also a critical regulator of mammary gland morphogenesis and luminal-cell differentiation. Asselin-Labat et al. (2007) demonstrated that the loss of *Gata3* leads to an expansion of luminal progenitor cells and a block in differentiation. Although *Gata3* mutant mice have disorders in numerous organ systems, the major cause of embryonic lethality seems to be a heart failure due to noradrenaline deficiency of the sympathetic nervous system (Lim et al., 2000). A paper describing additional malformations in *Gata3*-deficient heart development suggests that the short outflow tract and insufficient rotation of truncus arteriosus during looping may result in severe heart defects (Raid et al., 2009).

GATA2 and GATA3 often have overlapping expression pattern in various cell types suggesting compensatory mechanisms in different tissues. In many organs, however, GATA factors serve diverse functions, like in the blood. Table 1 shows a list of GATA2 and GATA3 involvement in different developmental processes, excluding inner ear and CNS, which are discussed in more detail in chapter 4.5.

Table 1. Involvement of GATA2 and GATA3 in diverese processes.

Tissue \ Factor	GATA2	GATA3
Blood	Proliferation and survival of hematopoietic stem and progenitor cells (Tsai and Orkin, 1997)	Differentiation of T helper type 2 cells (Zheng and Flavell, 1997; Ho et al., 2009)
Peripheral nervous system	Differentiation of sympathetic neurons (Tsarovina et al., 2004)	Survival of sympathetic neurons (Tsarovina et al., 2010)
Skin	Proliferation of mast cells (Jippo et al., 1996)	Cell lineage determination of the multipotent progenitor cells (Kaufman et al., 2003).
Kidney	Morphogenesis of ureter, bladder (Zhou et al., 1998)	Proliferation and guidance of the nephric duct; development of kidney, ureter (Grote et al., 2006; 2008)
Genital	Morphogenesis of sex organs (Zhou et al., 1998)	Development of genital tracts (Grote et al., 2008)
Mammary gland	unknown	Morphogenesis and luminal cell differentiation (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2008)
Heart	unknown	Development of outflow tract (Raid et al., 2009)
Bone	Generation of osteoclast progenitors (Yamane et al., 2000)	unknown
Parathyroid gland	unknown	Differentiation and survival of progenitor cells (Grigorieva et al., 2010)
Placenta	Differentiation of trophoblast giant cells (Ng et al., 1994)	
Prostate	Regulation of androgen-dependent transcription (Böhm et al., 2009)	
Pituitary gland	Proliferation and differentiation of gonadotropes and thyrotropes (Charles et al., 2006)	
Adipose tissue	Negative regulation of adipocyte differentiation (Okitsu et al., 2007)	

1.6.2 GATA2 and GATA3 in inner ear

Gata2 and *Gata3* are the only family members known to be expressed in the inner ear. *Gata3* is expressed in the whole mouse otic placode epithelium at E8.0 (Lawoko-Kerali et al., 2002), whereas *Gata2* expression is first detected after the otic vesicle closure at E9.5 (Lilleväli et al., 2004). The expression of these two *Gata* genes is highly overlapping at E10.5, but becomes increasingly distinct in later stages. *Gata2* is mainly restricted to the dorsal nonsensory vestibular epithelium and vestibulocochlear ganglion, whereas *Gata3* is predominantly expressed in the ventral cochlear duct and ganglion (Lilleväli et al., 2004).

Gata2 knockout embryos do not exhibit any morphological defects in the otic vesicles at E10.5 (Lilleväli et al., 2004). The absence of the inner ear phenotype in *Gata2* mutants could be caused by *Gata3* expression in these vesicles compensating the loss of *Gata2* during early otic development. On the other hand, Karis et al. (2001) reported that the development of *Gata3*-deficient mouse ears arrest at otic vesicle stage, the outgrowth of both the semicircular ducts and the cochlear duct is affected and the only outgrowing part is the endolymphatic

duct. Lilleväli et al. (2004) showed that the developmental arrest of *Gata3*-deficient inner ear is accompanied by the strong delay and decrease in *Gata2* expression at these early stages. This observation suggested that these two GATA factors act in the same genetic pathway, and that *Gata2* is downstream of *Gata3* in the inner ear.

The development of the inner ear has been further examined in *Gata3*-knockout embryos. *Gata3* is specifically expressed in the cochlear ganglion neuroblasts in the ventral domain of the otic vesicle. At subsequent stages, the expression of *Gata3* may distinguish cochlear ganglion neurons from the vestibular ones, and it selectively regulates *Neurod1* expression and maintenance during cochlear neuroblast migration (Karis et al., 2001; Lawoko-Kerali et al., 2004). Additionally, *Gata3* is involved in the pathfinding of efferent neuron axons from the rhombomere 4 neuron population into the otic sensory epithelia (Karis et al., 2001).

GATA3 also has an essential role in the maintenance of cochlear hair cells in adult animals. Heterozygous *Gata3* mice show significant morphological degeneration of hair cells and supporting cells in the organ of Corti, causing progressive hearing loss (van der Wees et al., 2004). Similarly, haploinsufficiency of the human *Gata3* gene causes HDR syndrome, characterized by deafness (van Esch et al., 2000).

1.6.3 GATA2 and GATA3 in nervous system

Among the six GATA family members only *Gata2* and *Gata3* expression has been reported in the central nervous system (Nardelli et al., 1999). *Gata2* and *Gata3* are expressed in numerous types of neural progenitor cells as well as in the post-mitotic neurons (Nardelli et al., 1999). Both *Gata2* and *Gata3* expression is initiated in the neural tube between E9.0-11.5, when many neuronal subtypes are born. The expression of the two GATA factors overlaps in the developing CNS in motor neuron and ventral interneuron precursors (Nardelli et al., 1999). GATA2 participates in the differentiation pathway of different types of neurons in the ventral hindbrain and spinal cord, namely cranial motoneurons (Nardelli et al., 1999; Pata et al., 1999), serotonergic neurons (Craven et al., 2004), and interneurons (Zhou et al., 2000; Karunaratne et al., 2002). El Wakil et al. (2006) suggests that GATA2 acts as a negative regulator of proliferation in neural progenitor cells.

In the embryonic brain, *Gata3* expression is restricted to the diencephalon at E10.5, and is localized in the mesencephalon and parts of the pons by E11.5 (Oosterwegel et al., 1992; George et al., 1994). *Gata3* mutant brains have smaller and collapsed ventricles, and a thinned, highly disorganized neuroepithelial layer (Pandolfi et al., 1995). Pata et al. (1999) showed that in rhombomere 4 the expression of *Gata3* is dependent on GATA2, which in turn is under the positive control of HOXB1. The loss of *Gata3* expression inhibits the migration of facial branchiomotor neurons to rhombomere 6. In distinct brain regions, the expression of *Gata3* is often dependent on the presence of GATA2, suggesting that GATA3, in contrast to the situation in inner ear, acts downstream of *Gata2* in the developing CNS (Nardelli et al., 1999; Pata et al., 1999).

One of the major brain regions expressing *Gata3* in the adult stage is the midbrain raphe system (Zhao et al., 2008). In the caudal raphe nuclei GATA3 is essential for the proper development of the serotonergic neurons and possibly for their role in locomotion (van Doorninck et al., 1999). The expression of *Gata2* has also been detected in adult midbrain, whereas its expression is not detected in serotonergic neurons suggesting different functions for GATA factors in adult midbrain (Nozawa et al., 2009).

2 Aims of the study

Gata2 and *Gata3* are expressed during mouse inner ear development in a partially overlapping manner (Lilleväli et al., 2004). The inactivation of mouse *Gata3* causes severe problems in inner ear morphogenesis. The otic vesicle development arrests and more advanced structures are unable to form (Karis et al., 2001). In contrast, *Gata2*-deficient otic vesicles display no morphological defects before the mutant embryos die at E10.5 (Lilleväli et al., 2004). In the developing mouse CNS, GATA2 has been shown to regulate the neuronal subtype specification (Zhou et al., 2000; Craven et al., 2004). Although *Gata2* expression was previously described in the murine midbrain, the cell type specificity remains unknown (Nardelli et al., 1999). Thus, several questions regarding the roles of these two GATA factors in inner ear and CNS development were unanswered when this study was initiated.

The detailed aims of this study were:

1. To gain indications of the potentially conserved functions of GATA2 and GATA3 in mouse and chicken inner ear development with a comparative spatio-temporal expression analysis of the two *Gata* genes.
2. To understand the biological function of GATA3 during mouse otic vesicle formation.
3. To identify target genes for GATA3 in mouse inner ear morphogenesis.
4. To examine the role of GATA3 in vestibular and cochlear sensory differentiation in pharmacologically rescued mouse embryos.
5. To identify the biological function of GATA2 in inner ear development using the conditionally inactivated *Gata2* embryos.
6. To perform an initial analysis of the role of GATA2 in mouse midbrain.

3 Materials and methods

Tables include genetically modified mouse lines, experimental methods, antibodies, and RNA probes personally applied in this study. Roman numbers indicate the respective publications where they are described in detail.

Table 2. Genetically modified mouse lines used in this study.

Transgenic allele	Publication
Foxg1-Cre	IV
Gata2 flox	IV, V
Gata3-/- (*Gata3nlslacZ/nlslacZ*)	II, III
Rosa26 (*R26R*)	IV

Table 3. Experimental methods used in this study.

Method	Publication
„Rescue' of *Gata3-/-* embryos	III
Cell proliferation assay	II, IV
Detection of programmed cell death	II, IV
Generation of *Gata2* flox-allele	IV
Immunohistochemistry	III, IV, V
In ovo electroporation	V
Paint-filling of inner ears	IV
Radioactive RNA *in situ* hybridisation	I, II, III, IV, V
Semi-thin sectioning	III
Statistical methods (Student's t-test)	II, IV
Whole mount RNA *in situ* hybridisation	I, II, IV
X-gal staining	II, III, IV

Table 4. Primary antibodies used in this study.

Raised in	Antigen	Dilution	Publication
goat	HA	1:500	V
mouse	BrdU	1:400	II, III, IV
	calretinin	1:200	III
	EphB2	1:200	IV
	Lim1/2 (Lhx1)	1:10	V
	Gata3	1:200	III
rabbit	Bcl-X	1:200	IV
	Gata2	1:250	III, IV, V
	Sox2	1:400	III
	Prox1	1:100	III

Table 5. RNA *in situ* hybridization probes used in this study.

Mouse probes	Publication
Atoh1	III, IV
Bmp4	II
Cdkn1b ($p27^{Kip1}$)	III, IV
Dach1	II
Dlx5	II, IV
Drapc1	II
Epha4	II
Etv5	II
Fgf10	II, IV
Fgf3	II
Fgfr2(IIIb)	II
Gad1	V
Gata2	IV, V
Gata3	III, V
Gbx2	II
Gjb2	II
Helt	V
Jag1	III, IV
Lfng	II
Netrin1	III, IV
Nr4a3 (*Nor1*)	IV
Otx2	V
Pax2	III
Pou4f3	III
Prrx2	IV
Six1	II
Wnt10a	II
Wnt2b	II
Wnt3a	II
Wnt6	II

Chicken probes	Publication
cBmp4	I
cFgf10	I
cGad1	V
cGata2	I
cGata3	I, V
cNgn2	V
cPax2	I
cSlc17a6	V

4 Results and discussion

4.1 Comparison of *Gata2* and *Gata3* expression during chicken inner ear development (I)

We performed a detailed comparative spatio-temporal expression analysis of *Gata2* and *Gata3* during chicken otic morphogenesis to get insight into their potential roles in avian otic development and to identify potential redundancy between the two GATA factors. The main observation was that the expression domains of *Gata2* and *Gata3* were highly overlapping in early otic cup and vesicle epithelium, but became more complementary during later morphogenesis.

The *Gata3* expression was already initiated at the preplacodal stage, whereas *Gata2* was first detected in the otic cup. Both *Gata* genes were expressed in the lateral domain of the chicken otic cup and vesicle epithelium suggesting a role in the otic vesicle closure event. Subsequently, *Gata2* and *Gata3* were expressed in the vestibule and cochlea, but *Gata2* was mainly detected in the nonsensory parts and *Gata3* predominantly in the sensory domains. Essentially, *Gata2* and *Gata3* were expressed in the semicircular ducts, however, the expression pattern was complementary so that *Gata2* was detected in the inner edges of the semicircular ducts, whereas *Gata3* in the outer edges. In mouse, *Nr4a3* expression in the inner edges of the ducts is required for the proliferation of the nonsensory epithelium to maintain normal duct growth (Ponnio et al., 2002), and *Dlx5* in the outer edges of the semicircular ducts controls positional proliferation and apoptosis in the otic epithelium essential for the development of the three ducts (Merlo et al., 2002). According to the expression, GATA2 and GATA3 could also regulate proliferation or programmed cell death during avian vestibular development.

The expression in the mesenchyme surrounding the ducts suggests that the GATA factors might have a role in controlling the semicircular duct morphogenesis via regulation of mesenchymal-epithelial signaling. During morphogenesis the mesenchymal *Gata2* expression became restricted to the fibrocytes underlying the vestibular sensory organs, except the macula lagena. The fibrocyte cells are specialized in the regulation of inner ear fluid movement and ion homeostasis, which is essential for proper hair cell functioning (Delprat et al., 2002). Consistent with the expression, GATA2 could have a role in the control of fibrocyte development.

In chicken, *Gata2* was not expressed in the vestibular sensory organs, whereas *Gata3* was detected in the cristae and maculae. We found a restricted *Gata3* expression in striola of the utricular and saccular maculae (Fig.2 in I) that is a hook-shaped narrow area dividing the macula into the two parts with opposite hair cell polarity (Denman-Johnson and Forge, 1999). *Gata3* is expressed in the 6-10-cell-wide region in the center of the striola corresponding to the location where hair cell stereocilia undergo an 180˚ shift in orientation (Hawkins et al., 2003). Thus, GATA3 may have a role in defining the polarity of hair cells in the striola. Alvarado et al. (2009) proposed a model in which GATA3 regulates WNT, FGF, NOTCH, and BMP signaling pathways in avian utricular sensory epithelium and by that controls the specification of hair cell phenotype and/or axon guidance in the striola. These same signaling pathways are under the control of GATA3 in mouse skin epidermis and hair follicles, where it is required for differentiation and organization of hair follicles during skin development and regeneration (Kaufman et al., 2003; Kurek et al., 2007).

During the cochlear duct outgrowth, both *Gata2* and *Gata3* were expressed in the prosensory medial wall that gives rise to the basilar papilla, indicating a possible role in early cochlear sensory development. Overlapping expression of both *Gata* genes was detected in the tegmentum vasculosum and in the supporting cells of the basilar papilla, while only *Gata3*

was expressed in sensory hair cells (Fig.3 in I). *Gata3* expression is also maintained in the cochlear sensory epithelia in posthatch birds (Hawkins et al., 2003).

The complementary expression of the two *Gata* genes was also observed during otic ganglion development. We detected the expression of *Gata2* and *Gata3* in different compartments of the anteroventral otic vesicle domain, where the neuroblasts start to delaminate. At subsequent stages *Gata2* became dominant in the vestibular and *Gata3* in the cochlear ganglion (I). This observation is consistent with the notion that cochlear and vestibular neurons come from different compartments of the otic epithelium (Fekete and Wu, 2002; Bell et al., 2008). Jones and Warchol (2009) showed that *Gata3* was coexpressed with known neurogenic markers in the proneural-sensory domain of the chicken otic vesicle, and that *Gata3* was not expressed in migrating and proliferating neuroblasts, but was reestablished during differentiation of the cochlear neurons.

These combined results suggest possible redundant functions of GATA2 and GATA3 during chicken otic vesicle formation, while in later stages they seem to regulate diverse functions in cell-type specific manner in the vestibule and cochlea.

4.2 Analysis of the role of GATA3 in inner ear (I, II, III)

To better understand the role of GATA3 during inner ear development, we performed several studies. First, we compared the expression pattern of *Gata3* between birds and mammals with intent of detecting potential conserved functions of this factor in the two distinct vertebrate classes (I). Additionally, we performed a detailed study on the timing and molecular and cellular bases of the morphogenetic defects in the *Gata3*-deficient otic epithelium (II). We also checked the sensory organ development in *Gata3-/-* embryos (III).

4.2.1 Comparative expression analysis of *Gata3* during chicken and mouse otic development (I, II)

The comparison of *Gata3* mRNA expression in mouse (Lilleväli et al., 2004; Fig.1 in II) and chicken (I) revealed differencies in early stages of otic development, whereas highly conserved expression at subsequent stages was observed. Chicken *Gata3* was detected at the preplacodal stage covering a broad domain of the surface ectoderm including the presumptive otic region (Fig.1 in I), whereas mouse *Gata3* was first detected in an already distinguishable otic placode (Fig.1 in II). During the otic cup and vesicle stages, mouse *Gata3* was expressed throughout the otic epithelium (Lilleväli et al., 2004), while chicken *Gata3* was restricted to the lateral domain of the otic cup and vesicle (Fig.1 in II). Importantly, while mouse *Gata3* was strongly expressed in the ventromedial otic cup, closest to the hindbrain, no expression of chicken *Gata3* was detected there. This remarkably different expression pattern suggests at least two divergent roles for GATA3 during the early morphogenetic stages. In fact, we have shown that GATA3 has an especially important role during mouse otic epithelium invagination most likely related to its expression in the ventromedial cup (Fig.1 in II). Instead, the chicken factor may have a different role later, for example in otic vesicle closure according to its expression in the lateral domain near the closure area (Fig.1 in I). These results suggest that although the early morphogenetic events in chicken and mouse appear highly similar, the molecular control of these steps may not be conserved in vertebrates.

Gata3 expression became highly similar in mouse and chicken later when the otic vesicle started to elongate (Lilleväli et al., 2004; Fig.2 and 3 in I), suggesting similar and important functions in different vertebrate classes. In both species, *Gata3* was detected in the outgrowing semicircular duct epithelia and the surrounding periotic mesenchyme, as well as in the striola of the utricular and saccular macula. During maturation of the sensory crista,

Gata3 became specifically restricted to the nonsensory parts, namely the lingula region in chicken and the cruciate eminence in mouse. This conserved expression could have an essential role in the formation of crista. *Gata3* was also expressed in mammalian and avian cochlear sensory organs throughout their development and the mRNAs were detected in both sensory hair cells and supporting cells. This conservation of *Gata3* expression indicated an important role in the cochlear sensory development, which was confirmed in mouse (III). In addition, the expression of *Gata3* was detected in the mesenchymal cells surrounding the developing cochlear duct in both mouse and chicken embryos (Lilleväli et al., 2004; Fig.3 in I). Several studies have confirmed the importance of mesenchymal signaling for the cochlear morphogenesis using targeted mutagenesis of genes expressed in the periotic mesenchyme (Phippard et al., 1999; Kiernan et al., 2002; Xu et al., 2007; Trowe et al., 2010). Thus, the expression of *Gata3* in cochlear periotic mesenchyme could indicate an indirect regulation of cochlea development through the mesenchyme in mouse and chicken. Moreover, GATA3 could play an evolutionarily essential role in mesenchymal-epithelial signaling in different vertebrate species.

We found several interesting differences in *Gata3* expression between mouse and chicken otic ganglion (Lilleväli et al., 2004; I; II). *Gata3* was expressed throughout the mouse otic cup epithelium, including the whole ventromedial proneural domain, whereas the chicken factor was only partially overlapping with the proneural epithelium. At later stages, mouse *Gata3* was also detected in migrating and proliferating neuroblasts (Lawoko-Kerali et al., 2004), while *Gata3* was not present in these cells in chicken (Jones and Warchol, 2009; Fig.1 in I). During differentiation of the neurons, *Gata3* expression was progressively increased in both mouse and chicken cochlear ganglion (Lilleväli et al., 2004; Jones and Warchol, 2009; Fig.3 in I). Thus, while in mouse, *Gata3* is expressed throughout ganglion development and could play a role in specification, proliferation, migration, and differentiation of the cochlear ganglion neurons, the chicken counterpart appears to be mainly involved in the differentiation stage. The intriguing idea, however, would be that *Gata3* expression in the chicken otic cup epithelium labels presumptive cochlear ganglion neuroblasts prior delamination.

4.2.2 GATA3 is essential for normal morphogenesis during otic placode invagination in mouse (II)

The otic development is aberrant in *Gata3*-deficient mouse embryos (Karis et al., 2001). To better understand the developmental defects in *Gata3-/-* embryos at E8.5-11.5, we used a mouse line in which a *nlsLacZ* sequence is knocked-in to the *Gata3* locus and the activity of β-galactosidase reflected the endogenous expression of *Gata3* (Hendriks et al., 1999; Karis et al., 2001). Together with wild-type embryos, the heterozygous *Gata3+/nlsLacZ* (hereafter *Gata3+/-*) littermates were used as controls, because the inner ear development was morphologically normal in these embryos.

We detected *Gata3* expression in the otic placode at E8.5. This is earlier than the expression in the adjacent hindbrain starting at E9.0 (Nardelli et al., 1999; Pata et al., 1999), or in the periotic mesenchyme starting around E10.5 (Lilleväli et al., 2004). The otic placode formed without *Gata3*, but it had morphological defects at E8.5. The uniform thickness of the otic epithelium seen in control embryos was lost in *Gata3-/-* otic placodes, which had a thinner dorsal domain and thicker ventral part (Figure 7). At E8.75-9.0, the size of *Gata3-/-* otic cups was smaller, and the shape was different from the evenly round cup seen in control embryos. Moreover, a phenotypic variation occurred in the mutant cups. The *Gata3*-deficient otic cups were either elongated in the dorsoventral direction, or had an ectopic constriction in the medial wall (Figure 7). This morphological constriction formed a boundary dividing the otic cup epithelium into a smaller, thinner dorsal and a larger, thicker ventral region. In addition, the invagination process of the dorsal domain was often delayed compared to the

ventral region. During the otic cup stage, a close connection between the hindbrain and the ventromedial domain of the otic epithelium with the strongest *Gata3* expression was detected in control embryos. In contrast, the cup epithelium was more distant and separated by layers of mesenchymal cells in *Gata3*-/- embryos.

Although the otic placode and cup morphology was abnormal, the otic epithelium was able to close and form vesicle(s) in *Gata3* mutants. The mutant otic vesicles were either smaller or oval-shaped compared to the round control vesicles or divided into two separate structures. This division of the epithelium occurred along the ectopic morphological boundary observed at otic cup stage. Thus, lack of GATA3 causes defects in otic placode and cup morphology during the placode invagination process resulting in defective and sometimes disrupted otic vesicles.

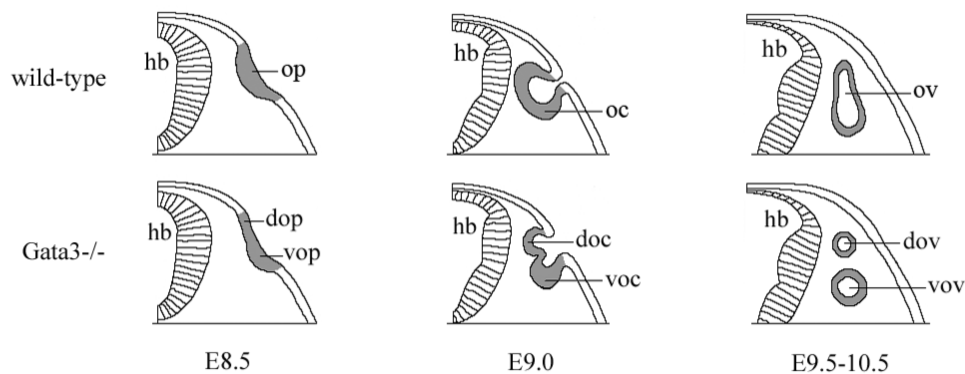

Figure 7. Early development of the wild-type and *Gata3-/-* otic region. At E8.5, the *Gata3*-deficient otic placode (op) has a thinner dorsal part (dop) and a thicker ventral part (vop). During otic placode invagination (E9.0), a constriction appears in the middle of the mutant otic epithelium, which divides the otic cup (oc) into the dorsal (doc) and the ventral (voc) regions. At E9.5-10.5, the otic epithelium closes to form the otic vesicle (ov), but in half of *Gata3-/-* embryos two vesicles form on one side or on both sides of the head. The dorsal vesicle (dov) is always smaller than the ventral (vov) one. hb, hindbrain.

One of the interesting questions is that why *Gata3* mutants show variable otic phenotypes. Although numerous studies have tried to find an answere for a phenomenon of variable pheotypes caused by single gene mutation, the underlying mechanisms are still poorly understood. Nevertheless, a number of factors have been suggested to cause variable phenotypes, such as modifier genes, environmental factors, allelic variation, and complex genetic and environmental interactions. For instance, the mutation in DFNB26 gene is known to penetrate incompletely causing in most cases deafness, whereas some individuals have normal hearing. Interestingly, a dominant modifier gene of DFNB26 was identified that suppresses deafness in homozygous individuals (Riazuddin et al., 2000). The exact mechanism how this modifier gene suppresses deafness is, however, not known. Whether an unspecified modifier gene regulates also *Gata3* remains to be elucidated. For example there could be a modifier gene that suppresses disruption of the *Gata3*-deficient otic epithelium and supports the development of an intact otic vesicle. Another possibility is that the mouse strain background could influence the phenotypic variability in *Gata3-/-* embryos. *Gata3* mutant line was under the inbred C57BL/6 background. The phenotypic variability has been described in transgenic mice generated in an inbred strain, but the widest range of phenotypes are caused by mixed backgrounds due to incomplete penetrance and variable expressivity of phenotypes (Doetschman, 2009). Finding the answere becomes even more complicated when

considering the fact that in half of *Gata3-/-* mutants the inner ear morphology on one side of the head is different from that observed on the other side of the head.

Despite the disruption of the otic epithelium into two parts, the dorsoventral patterning occurred normally in *Gata3-/-* otic vesicles. The dorsal *Dach1* and ventral *Six1* expressions were detected in corresponding domains of the otic epithelium in both controls and mutants. These results indicate that the initial specification of the dorsal fate by hindbrain-derived WNT signals and the ventral fate by SHH from the notochord (Riccomagno et al., 2005) occurs normally in the absence of *Gata3*.

These results suggest that GATA3 has a critical role in the otic placode invagination process. The aberrant morphogenesis of *Gata3*-deficient otic epithelium could result from altered development of adjacent hindbrain regions. *Gata3* is not expressed in hindbrain during otic placode invagination, however, suggesting that the observed otic phenotype originates from intrinsic problems in the otic epithelium, such as increased adhesion and reduced intraepithelial migration.

4.2.2.1 Hindbrain and otic placode invagination (II)

The morphological division of the otic cup that leads to the disruption of the otic epithelium into two vesicles in *Gata3-/-* embryos was a novel surprising and interesting finding. Ectopic otic vesicles also form in some mouse mutants in association with the aberrant hindbrain development (Fekete, 2004).

The molecular mechanism of the otic placode invagination is poorly understood process. A close attachment of the otic epithelium to the hindbrain basal lamina, however, may be important for normal otic placode invagination in chicken (Moro-Balbás et al., 2000; Visconti and Hilfer, 2002). Inactivation of *Gata3* leads to an increased distance between the ventromedial part of the otic cup and hindbrain, whereas the dorsal otic cup domain remained at least partially in close contact with the neural tissue and thus, was most likely susceptible to hindbrain-derived signals. The reduced contact to the hindbrain basal lamina suggests that GATA3 positively controls the expression of certain extracellular matrix molecules and/or their receptors, such as laminins, integrins, heparan sulphates, or N-CAM thought to be involved in attachment (Brown et al., 1998; Moro-Balbás et al., 2000; Visconti and Hilfer, 2002). We could not, however, detect any significant decrease in the expression levels of these extracellular matrix components in our comparative microarray assay that was carried out between control and *Gata3-/-* otic regions at E9.5.

More recently, Barrionuevo et al. (2008) identified a second mouse gene, *Sox9* that was important for otic invagination. In their study, the otic placode invagination problems were also accompanied by reduced association between the otic epithelium and the hindbrain. In their elegant experiments with mosaic inactivation of *Sox9* in some of the placode cells, however, indicated that groups of *Sox9*-positive cells invaginated and formed micro-vesicles even without any contact to the hindbrain. These findings suggest that the deficient attachment of the otic epithelium to the neural tube is not the primary reason for the aberrant otic placode invagination and subsequent vesicle formation observed in *Gata3* and *Sox9* mutant mice. In addition, they indicate that the contacts between otic epithelium and hindbrain are not as important during mouse otic invagination as thought from those performed in chicken embryos. Furthermore, transplantation studies in quail/chicken chimeras have shown that otic placodes can also invaginate in birds, although placed far away from the neural tube (Groves and Bronner-Fraser, 2000).

4.2.2.2 Cell-cell adhesion in early otic epithelium (II)

We carried out a comparative large-scale microarray analysis between wild-type and *Gata3-/-* mRNA populations isolated from E9.5 closed otic vesicles to gain information about potential

GATA3 target genes. We used the microarray that permitted to measure the expression level of more than 36,000 mouse genes and expressed sequence tags (EST). The analysis allowed us to identify 250 upregulated genes and 63 downregulated genes using an established empirical threshold. Changes were detected especially in several genes encoding proteins involved in mediating cell adhesion and motility. The highest upregulation (5.7-fold) was detected in *Gjb2* mRNA level in the absence of *Gata3*. In addition, the validation of the result with the RNA *in situ* hybridization also showed that *Gjb2* expression was activated precociously and ectopically in *Gata3-/-* otic cups and in the newly closed otic vesicles at E9.0-9.25, while in control embryos expression was first detected in sensory patches around E10.5. *Gjb2* encodes for CONNEXIN 26, which has a widely known function in cell-cell communication, however, interestingly connexins have also a gap junction-independent function in cell adhesion and motility (Wei et al., 2004). In addition, we found that *Epha4* and *Ephb4* mRNA levels were upregulated in otic vesicles in the absence of *Gata3*. Previous studies demonstrated that members of the Eph receptor tyrosine kinase family and their ligands, ephrins, have essential roles in the regulation of cell adhesion and migration during development (Klein, 2004; Poliakov et al., 2004). In contrast, an extracellular matrix protein coding gene *Secreted phosphoprotein 1* (*Spp1*) was four times decreased in the mRNA level in the *Gata3-/-* otic region. SPP1 mediates cell adhesion and cell-matrix interactions (Wai and Kuo, 2004). These results strongly suggest that adhesion properties and cell motility could be changed in *Gata3*-mutant otic vesicle epithelium.

Two GATA factors in *Drosophila*, grain and pannier, control the organ shape by regulating intraepithelial motility and adhesion (Calleja et al., 2000; Brown and Castelli-Gair Hombría, 2000). We propose that GATA3 could have an equivalent role in mammalian otic epithelium. Our expression analysis results in the otic vesicle and morphological analyses during the invagination process suggest that the increased intraepithelial adhesion leads to a decrease in epithelial motility.

Fate-mapping experiments in chicken have demonstrated that the endolymphatic duct outgrowth is largely accomplished by migration of cells from the ventral part of the otic epithelium (Brigande et al., 2000a). Such migration seems to be altered in *Gata3*-deficient otic epithelium with an ectopic morphological constriction leading to a situation where ventral cells cannot efficiently contribute to the presumptive dorsal endolymphatic duct domain. The ectopic constriction in *Gata3*-mutant otic epithelium also corresponded to the ventral expression border of several dorsally expressed genes that separated the epithelium into the dorsal and ventral areas. In contrast to the equally thick and evenly proliferating otic cup epithelium in control embryos, *Gata3* mutants often had a thicker ventral otic cup region that contained several layers of proliferating cells and a thinner dorsal otic cup region containing areas that lacked proliferating cells. This abnormal distribution of dividing cells in *Gata3-/-* otic epithelium could indicate reduced cell migration from ventral to dorsal domain of the otic cup. It could be that the stronger cell-cell contacts in *Gata3-/-* epithelium could contribute to the inefficient movement of the normally *Gata3*-expressing ventral cells to the dorsal domain.

Interestingly, the loss of SOX9 also leads to changes in the adhesive properties of the otic epithelium, although the effect was already observed in the otic placode. The cell-cell contacts in *Sox9-/-* embryos were reduced and the adhesion-mediating transmembrane receptor gene *Epha4* was downregulated. The observed reduction in epithelial adhesion was thought to be the main reason behind the problems in placode invagination (Barrionuevo et al., 2008). In addition, the absence of *Pax2* in chicken otic region also leads to the loss of cell adhesion molecules N-cadherin and N-CAM and as a consequence, the placodal cells lose apical cell-cell contacts and the placode fails to invaginate (Christophorou et al., 2010). These results suggest that the adhesion properties of otic placode cells can be considered highly or even the most important determinants in placode invagination.

Importantly, while changes in adhesion properties were already detected in the otic placode epithelium in both *Sox9-/-* and *Pax2-/-* embryos, the upregulation of *Epha4* and *Ephb4* expression in *Gata3* mutants was discovered after otic placode invagination, in closed otic vesicles. Since the first morphological defects in *Gata3-/-* epithelium could already be observed in the placode stage, it is highly likely that the same genes are affected earlier, during otic placode invagination.

4.2.3 GATA3 is a direct regulator of *Fgf10* in mouse inner ear (II)

We noticed that the expression domains of *Fgf3* and *Fgf10* were considerably overlapping with *Gata3* in the anteroventral region of the otic vesicle and in the cells forming the vestibulocochlear ganglion. While the expression of *Fgf3* appeared unchanged in *Gata3-/-* embryos, *Fgf10* could not be detected at E9.5 in otic epithelium or the ganglion by *in situ* hybridization and microarray analysis. Therefore, we focused on the possibility that GATA3 may directly control the expression of *Fgf10* in the early otic epithelium.

Several conserved GATA3 binding sites were identified in the *Fgf10* upstream region in mouse, human, and chicken genomic DNA (Fig.7 in II) by using the Consite algorithm (Lenhard et al., 2003). We mapped the functional GATA3 binding sites between -3410 and -1659 in the *Fgf10* upstream region (Fig.7 in II) that also includes the enhancer sequence regulating specific expression of *Fgf10* in mouse inner ear (Ohuchi et al., 2005). To better understand the transactivation capacity of GATA3, we cloned the upstream region containing the conserved GATA3 binding sequences from *Fgf10* into a firefly luciferase reporter vector and transfected it into NIH3T3 cells with or without the *Gata3* expression vector. Accordingly, cotransfection with a GATA3 plasmid transactivated the reporter gene approximately twofold, suggesting that GATA3 could efficiently bind to the GATA3 binding sites present in the *Fgf10* regulatory region. These results identify *Fgf10* as the first potential target for GATA3 in mouse otic epithelium.

Gata3 and *Fgf10* are both expressed in the vestibular sensory regions during semicircular duct outgrowth (Lawoko-Kerali et al., 2002; Pauley et al., 2003; Lilleväli et al., 2004). Using a chicken model, Chang et al. (2004) proposed that FGF10 secreted from the sensory epithelium regulates the outgrowth of the semicircular ducts by upregulating *Bmp2* expression in the duct genesis zones. Thus, the lack of semicircular duct outgrowth in *Gata3*-deficient mouse embryos could partially result from the missing *Fgf10* expression of the vestibular sensory epithelia.

FGF10 is required to promote the expression of various proneural and neurogenic genes in vestibulocochlear ganglion precursor cells (Alsina et al., 2004). Since the neuronal development in *Fgf10* mutants occurred normally, the additional FGF family member, FGF3 could probably substitute FGF10 in the otic epithelium. The expression of *NeuroD* is positively regulated by GATA3 in mouse migrating cochlear neuroblasts (Lawoko-Kerali et al., 2004). Our microarray also showed that the expression level of *NeuroD* decreased in the *Gata3-/-* otic region at E9.5 (Table1 in II). Thus, GATA3 could regulate the expression of *NeuroD* at least partially via FGF10-signaling in cochlear neuroblasts.

4.2.4 Sensory organ primordium is established in the absence of *Gata3* (II, III)

Gata3 is expressed in the prosensory domain of the otic vesicle epithelium and continues to be expressed in all inner ear sensory epithelia except the macula of saccule (Karis et al., 2001; Lilleväli et al., 2004). After E14.5, *Gata3* becomes downregulated in the vestibular sensory epithelia, but the expression persists throughout the development in the striolar region of the maculae (Karis et al., 2001; Lawoko-Kerali et al., 2002; Lilleväli et al., 2004). Unlike the vestibular sensory epithelia, the expression of *Gata3* is maintained in the cochlear prosensory

epithelium as well as in the post-mitotic hair and supporting cells (Lilleväli et al., 2004; van der Wees et al., 2004; Fig.4 in III).

The initial determination towards the sensory fate occurred without GATA3 illustrated by the expression of *Lfng* and *Bmp4* at E9.5-10.5 (Fig.4 in II). Instead, *Fgf10* was the only early sensory marker tested whose expression was downregulated in *Gata3-/-* embryos at E9.5 as well as E11.5. The decrease of *Fgf10* in *Gata3*-deficient otic epithelium, however, is not likely to interfere with the general sensory development since the formation and differentiation of most sensory regions is largely unaffected in *Fgf10-/-* mice (Pauley et al., 2003).

4.2.5 Variable morphological problems and failure to form differentiated cells in the cochlea in *Gata3*-deficient embryos at E11.5-17.5 (III)

To gain new information about the morphological development and hair cell differentiation of *Gata3*-deficient inner ears after E11.5, we produced drug-rescued mutant embryos. The early embryonic lethality of *Gata3-/-* mutants was overcome by a mixture of special drugs that supported the development of organs depending on adrenergic innervation (Lim et al., 2000; Kaufman et al., 2003). By this method *Gata3*-deficient embryos survived at least up to E17.5. Recently, Maison et al. (2010) showed that this kind of catecholamine intermediate treatment as such does not have any effect on mouse inner ear development.

As described before, inactivation of *Gata3* leads to aberrant and variable otic phenotypes, where either one small intact oval shaped vesicle is formed or the otic epithelium becomes disrupted and divided into two vesicles with different identities (II). The *Gata3-/-* inner ear morphology was accordingly variable between E11.5-16.5. When the *Gata3*-deficient otic epithelium was divided into dorsal and ventral vesicles, a more severe inner ear phenotype was observed also at later stages. The development of the dorsal vesicle arrested completely whereas the ventral vesicle showed some growth, forming a larger cavity with variable forms, but without any distinguishable otic compartments. In addition, morphologically distinct sensory organs were not detected, although thickened epithelium was observed in the dorsal portion of these vesicles. This epithelium was able to adopt a sensory fate and to express *Jag1* and *Sox2* and to produce some hair cells indicated by the weak expression of *Atoh1* and *Pou4f3* (III).

The intact *Gata3-/-* otic vesicles showed a less severe phenotype usually with distinct vestibular and cochlear compartments and a small endolymphatic duct, but the three semicircular ducts were always lost in the absence of GATA3 (Karis et al., 2001; III). Despite the lack of semicircular ducts, two crista-like structures could be observed at E14.5-16.5 in *Gata3*-deficient ears. However, the *Gata3-/-* cristae were smaller in size, had fewer cells and lacked a nonsensory cruciate eminence structure suggesting that GATA3 is required for the morphological development of the cristae. Also the hair and supporting cell production appeared inefficient in the *Gata3-/-* cristae, indicating that the early expression of *Gata3* in the presumptive crista epithelia may be necessary to produce a sufficient number of sensory progenitors that differentiate into hair and supporting cells. A similar reduction of sensory cells was observed also in the utricular and saccular maculae. However, the saccular macula was less affected than the utricular macula, which is in line with the fact that *Gata3* is not expressed in the saccule during mouse inner ear morphogenesis (Lilleväli et al., 2004).

An interesting thing was observed in some of the intact *Gata3-/-* ears where the utricular and saccular compartments did not separate properly. In these ears with only two compartments (vestibular and cochlear), the vestibular sensory epithelia remained undivided. This suggests that compartmentalization is a major driving force for the sensory epithelium separation into distinct sensory organs. Since this phenotype was not always observed in *Gata3-/-* embryos it is likely to be a secondary effect due to the more general problems in

morphogenesis and growth in the otic epithelium. The sensory epithelia segregation is in fact thought to depend on correct specification of both sensory and nonsensory epithelia and this could be controlled by a large number of genes (Bok et al., 2007).

The *Gata3-/-* cochlear duct grew ventrally in the less severely affected ears, but remained shorter and relatively uncoiled. The thick cochlear wall expressed *Jag1* at E14.5-15.5, but none of the other sensory area marker genes including *Sox2*, *Prox1*, *Cdkn1b*, *Atoh1*, and *Pou4f3*. This demonstrated that although the cochlear sensory epithelium was initially specified, no hair or supporting cells formed without GATA3 (Fig.4 in III). These results also suggested that *Jag1* expression is independent of GATA3. In addition, the resent observation that SOX2 and CDKN1B are present in *Jag1-/-* cochleae (Basch et al., 2011) suggests that JAG1 and GATA3 may act on different pathways in controlling cochlear sensory development. A similar *Gata3* cochlear phenotype was recently characterized by Duncan et al. (2011) who investigated gene expression changes at E12.5 inner ears. They also showed that the projections of the vestibulocochlear efferent neurons that normally express *Gata3* are disrupted in *Gata3-/-* embryos.

The loss of *Sox2* expression in *Gata3-/-* sensory epithelium at E14.5-15.5 suggested that GATA3 acts on the same pathway with SOX2 in the cochlea during hair and supporting cell differentiation (Figure 8). The expression of the cell proliferation inhibitor *Cdkn1b* and the hair cell differentiation marker *Atoh1* was lost in both *Gata3* (Fig.2 and 4 in III) and *Sox2* mutants (Kiernan et al., 2005a) and SOX2 is known to activate the sensory marker *Prox1* (Dabdoub et al., 2008) that is also missing in *Gata3-/-* sensory epithelium. These results suggested that GATA3 may act upstream of SOX2, ATOH1 and PROX1 during prosensory domain specification and cochlear sensory epithelium differentiation. In addition, it appears that without GATA3 the sensory progenitors are unable to upregulate *Cdkn1b* expression indicating problems in the withdrawal of cell cycle and establishment of the prosensory domain. Based on these observations, a molecular cascade in cochlear development is proposed in Figure 8.

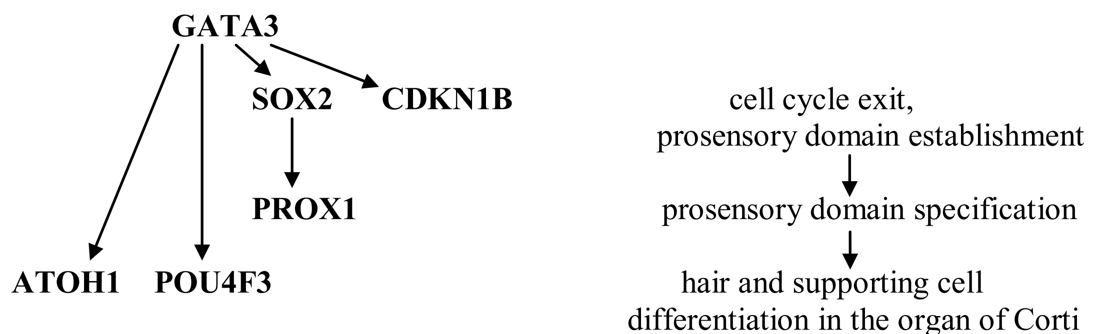

Figure 8. A schematic representation of the hypothesis of the GATA3 dependent molecular cascade controlling cochlear sensory differentiation. GATA3 appears to be necessary for the sensory progenitors to exit the cell cycle at E12.5-E14.5 and to upregulate *Cdkn1b*. GATA3 is also required to activate expression of *Sox2* known to specify the prosensory domain (E13.5-14.5) and for the upregulation of *Prox1*. Further differentiation of cochlear sensory epithelia (E15.5-18.5) involves genes such as *Atoh1* and *Pou4f3* that are not expressed in *Gata3-/-* cochleae.

GATA3 is required for inner ear development from the first morphological event, invagination of the otic epithelium (Fig.1 in II) that could also affect the later development and cellular differentiation in different otic sensory organs. However, inactivation of *Gata3* affects more cochlear than vestibular sensory organ formation that could be related to the downregulation of *Gata3* expression in vestibular sensory epithelium in wild-type embryos

around E14.5 (Lilleväli et al., 2004). When vestibular sensory cell differentiation occurred in *Gata3*-deficient embryos, then cochlear progenitor cells failed to differentiate into sensory cells and the epithelium remained immature. Similarly, inactivation of *Gata3* is known to block differentiation of progenitor cells in the skin (Kaufman et al., 2003) and mammary gland morphogenesis (Asselin-Labat et al., 2007).

Curiously, the undifferentiated *Gata3*-deficient cochlear sensory epithelium started to become thinner at E15.5 until it formed an expanded sac-like and thin-walled structure at E16.5. However, this is very different to what has been described in skin and mammary glands of *Gata3-/-* embryos where an expansion of hair follicle and luminal progenitor cells, respectively, could be observed (Kaufman et al., 2003; Asselin-Labat et al., 2007). Although the inability to induce *Cdkn1b* expression and presence of proliferating cells in *Gata3-/-* cochlear sensory epithelium could result in the expansion of sensory progenitor cells, this was not observed. Instead, a thinning of the cochlear sensory epithelium was detected. This could be due to extensive cell death and degeneration of the cochlear region that counteract the increased proliferation. Indeed, an increased number of apoptotic cells in *Gata3-/-* cochlear epithelium have been detected during the sensory organ formation (Duncan et al., 2011). A similar cochlear thinning has been described in *Pax2-/-* embryos where increased cell death in cochlear mesenchyme and epithelium was observed (Bouchard et al., 2010). Moreover, these mutants lacked otic capsule bone in medial regions next to the brain (Bouchard et al., 2010). Missing otic bone around the affected cochleae was also observed in *Gata3* mutants (Fig.2 in III) suggesting that GATA3 might be essential for cell survival in both otic epithelium and mesenchyme.

4.3 Analysis of the role of GATA2 in inner ear (I, IV)

The expression of *Gata2* is initiated in the lateral wall of the newly closed otic vesicle at E9.5 in mice. At E10.5 the expression becomes stronger in the dorsal nonsensory vestibular epithelium and the vestibular ganglion. In addition, *Gata2* expression is confined to the cochlear nonsensory epithelium and stria vascularis (Lilleväli et al., 2004). The previous analysis of inner ear development in *Gata2-/-* embryos did not detect any morphological defects in the otic vesicles before mutant death at E10.5 (Lilleväli et al., 2004). We used a conditional mutagenesis approach to address the later role of GATA2 during inner ear development (IV). Similarly to *Gata3*, to identify potential conservation between diverse species we compared the expression of *Gata2* in mouse (Lilleväli et al., 2004; IV) and chicken (I) otic development.

4.3.1 Comparative expression analysis of *Gata2* during chicken and mouse otic development (I, IV)

The comparison of mRNA expression identified similar expression domains for *Gata2* during mouse and chicken otic development. *Gata2* expression was first observed in the chicken otic cup, while in the mouse it appeared in the newly closed otic vesicle. Consequently, an earlier initiation of both *Gata2* and *Gata3* expression was detected in chicken relative to the morphogenetic stage of the otic epithelium suggesting that chicken factors may play an even earlier role in inner ear development than the mouse counterparts. *Gata2* expression was highly similar in both species being detectable in the lateral domain of the otic vesicle epithelium.

4.3.1.1 The vestibule (I, IV)

During vestibular development, *Gata2* was predominantly and continuously identified in the nonsensory epithelia and in the mesenchymal fibrocytes underlying the vestibular sensory organs in both mouse and chicken.

During semicircular duct development in chicken, *Gata2* was restricted to the proximal parts contributing to the fusion plates. After fusion plate clearance, expression was detected in the inner edges of the newly formed semicircular ducts. Unlike the restricted expression of *Gata2* in chicken semicircular duct epithelium (Fig.2 in I), however, the mouse counterpart was detected over the whole developing duct epithelium (Lilleväli et al., 2004; Fig.3 and 6 in IV). Thus, GATA2 could regulate a specific event, such as fusion plate formation during chicken semicircular duct development, while mouse GATA2 could be required for a more general morphogenetic process during semicircular duct formation (IV).

Gata2 was not expressed in any of the chicken vestibular sensory epithelia similar to that observed in mouse embryos indicating that GATA2 is not directly involved in vestibular hair cell development. Strong *Gata2* expression, however, could be detected in the mesenchymal fibrocytes underlying most of the vestibular sensory organs in both the chicken and mouse (Lilleväli et al., 2004; Fig.2 in I; Fig.3 in IV). Thus, GATA2 could influence hair cell function indirectly through the control of fibrocyte development known to be important for inner ear fluid movement and ion homeostasis (Delprat et al., 2002).

In the otic ganglion, *Gata2* expression was spatially conserved in the vestibular ganglion in both mouse and chicken. Chicken *Gata2* expression was already initiated in the migrating neuroblasts, however, while mouse *Gata2* appeared later in the vestibular compartment of the forming ganglion. Accordingly, GATA2 could have an earlier role in chicken, the migration of neuroblasts, and a later role in mouse, the differentiation of the otic ganglion cells. The expression of *Gata3* was spatially and temporally different to *Gata2* during ganglion formation in both mouse and chicken.

4.3.1.2 The cochlea (I)

In the outgrowing cochlear duct of both mouse and chicken, we detected *Gata2* in the nonsensory as well as in the prosensory walls. At later stages, during differentiation of the sensory epithelium, *Gata2* expression was confined to certain supporting cells of the basilar papilla and organ of Corti, whereas no expression could be detected in the hair cells (Lilleväli et al., 2004; Fig.3 in I). This implies that GATA2 is not directly involved in cochlear hair cell maintenance. The expression of *Gata2* in the stria vascularis in mouse and the tegmentum vasculosum in chicken (Lilleväli et al., 2004; Fig.3 in I), however, may indicate an indirect role in hair cell maintenance via regulating endolymph production and homeostasis.

Altogether, the expression of *Gata2* was highly conserved during inner ear development in mouse and chicken, although some temporal differences emerged.

4.3.2 Growth of semicircular ducts is regulated by GATA2 (IV)

We raised the question of whether GATA2 has a role in later otic morphogenesis and to answer this, we generated a *Gata2* conditional LoxP-sites-containing allele (*Gata2*$^{fl/fl}$) and used the *Foxg1-Cre* mouse line (Hébert and McConnell, 2000) to perform an inactivating deletion in the *Gata2* gene. In our hands, the *Foxg1* driven *Cre* recombination occurred in a reporter mouse line by E10.5, at a stage where no phenotype could be observed in the conventional *Gata2-/-* embryos. We verified that *Gata2* expression was lost in inner ear epithelium and the surrounding mesenchyme at E10.5 and E14.5 (Fig.3 in IV).

Using histological and gene expression analyses as well as paint-filling of inner ears, we observed that otic development was not deficient in *Foxg1-Cre;Gata2*$^{fl/fl}$ (hereafter *Gata2*^{cko}) embryos before E14.5 despite the strong expression of *Gata2*, especially in the vestibular and

cochlear nonsensory epithelium all through the development. Surprisingly, the semicircular duct pouch outgrowth, fusion plate formation, and clearance of the fusion plate occurred normally in *Gata2^{cko}* mutants despite it being expressed in the duct epithelium during these critical events for duct formation. The diametrical growth of the superior, lateral, and posterior ducts was arrested without *Gata2* around E14.5-15.5, whereas in control embryos the ducts continued to grow in diameter and cell number at E14.5-16.5 so that a dramatic decrease in duct sizes could be observed at P0 between the control and *Gata2* mutants (Fig.5 in IV). The relatively late otic phenotype coincided with the downregulation of *Gata3* expression from the vestibular nonsensory epithelium around E14.5 (Lilleväli et al., 2004), suggesting that there could be functional redundancy between GATA2 and GATA3 at earlier stages.

To clarify the reasons behind the significant size reduction of the three ducts in *Gata2^{cko}* embryos, we verified whether cell proliferation or programmed death had changed. The highest cell proliferation rate in semicircular ducts was observed at E14.5, and it decreased during the next days (E15.5 and E16.5) in both the control and *Gata2* mutants indicating that the general tendency in the proliferation rate (gradual decrease during development) had not altered in *Gata2*-deficient ears. The proportion of proliferating cells was significantly smaller in all *Gata2^{cko}* ducts, however, at least at one of the analyzed time points between E14.5-E16.5 when compared to the controls. The reduction in proliferation rate appeared to occur at different developmental time points for the different ducts, which is in line with the previous observations that the three semicircular ducts have temporal differences in their development (Martin and Swanson, 1993). Next, we considered the possibility that an increased rate of programmed cell death could also be behind the reduced diameter of semicircular ducts in the absence of *Gata2*. We observed increased cell death only in the *Gata2^{cko}* lateral duct epithelium at E14.5, whereas no difference in the other two ducts or in the lateral duct at E15.5-16.5 could be detected between controls and *Gata2^{cko}* samples. The lack of differences between programmed cell death rates between most of the analyzed samples from controls and *Gata2^{cko}* embryos suggests that programmed cell death is not the major mechanism by which GATA2 controls duct size. Instead our results suggest that the influence of GATA2 on semicircular duct growth is mainly mediated through the control of cell proliferation.

4.3.3 Endolymph production and fluid homeostasis in *Gata2*-mutant embryos (I, IV)

In addition to the reduced cell proliferation rate in *Gata2^{cko}* semicircular duct epithelium, other reasons may lie behind the arrested growth of the three ducts. We analyzed the possibility that endolymph production and volume may be reduced in *Gata2^{cko}* inner ears. The otic phenotype in *Gata2^{cko}* embryos was very similar to that described in *EphB2* and *ephrinB2* mouse mutants showing decreased semicircular duct diameter due to reduced production of endolymph in the membranous labyrinth (Cowan et al., 2000; Dravis et al., 2007). *EphB2* is expressed in K(+)-secreting dark cells next to the vestibular sensory epithelia, and *ephrinB2* in the adjacent nonsensory transitional cells separating the dark cells from the hair cells (Cowan et al., 2000; Dravis et al., 2007). We found no presence of GATA2 in transitional or dark cells of the vestibular epithelium in control embryos, and accordingly, the inactivation of *Gata2* did not change the expression of *EphB2* in dark cells compared to the expression in wild-type embryos. Thus, it appears that GATA2 does not regulate the endolymph production in inner ear, at least not through EphB2-signaling in dark cells. Moreover, *Gata2* is not expressed in the endolymphatic duct and sac (Lilleväli et al., 2004) and we did not detect any swelling or reduction in the size of these structures in *Gata2^{cko}* mutants, further suggesting that GATA2 is not involved in controlling fluid homeostasis in the vestibular system.

Gata2 is also expressed in the mammalian stria vascularis and the avian tegmentum vasculosum of the cochlea (Lilleväli et al., 2004; Fig.3 in I) where the secretory marginal cells produce endolymph. No defects in the cochlear morphogenesis and cellular differentiation were, however, observed in *Gata2*-mutant embryos. The overlapping and conserved expression of *Gata3* in these cochlear parts in both mouse and chicken (I) suggest that GATA3 may compensate for GATA2 in some cochlear cells.

4.3.4 GATA2-dependent mesenchymal cell clearance in inner ear perilymphatic space (IV, unpublished data)

The perilymphatic space between the epithelial membranous labyrinth and outer mesenchyme-derived bony labyrinth becomes cleared from the inner mesenchymal cells to form a cell-free space filled with perilymph between E14.5-18.5 (Noramly and Grainger, 2002; Fig.4 and 6 in IV). The cellular and molecular mechanisms regulating the clearance process are not well understood. We identified GATA2 as the first factor known to control the removal of the mesenchymal cells from the vestibular perilymphatic space. Interestingly, the mesenchymal cells were removed normally from the cochlear perilymphatic space in the absence of *Gata2*, suggesting a diverse molecular control for perilymphatic space development of the vestibule and cochlea. This is in line with *Gata2* not being expressed in the cochlear perilymphatic mesenchyme.

The *Gata2* expression was detected in the inner mesenchymal cells surrounding the semicircular ducts and the utricle. In its absence the mesenchymal cell clearance was inefficient in both of these structures. Thus, GATA2 may have a direct cell-autonomous effect on mesenchymal cell clearance in the expressed regions. In this study, we concentrated on the formation of the semicircular duct perilymphatic space.

During the semicircular duct growth in the control embryos (E12.5-16.5), the perilymphatic mesenchymal cells were arranged in two populations separated by a thin membrane-like ring. The innermost mesenchymal cell population expressed high levels of *Gata2*, as did the membrane-like ring. On the contrary, the outermost cell population did not express *Gata2*. In the absence of *Gata2*, the inner mesenchymal cells were distributed fairly evenly in the perilymphatic space and were not able to form the ring-like structure. This finding suggests that GATA2 is required to form the ring structure. Since the outermost cells were not pushed towards the capsule in the *Gata2^{cko}* ears as efficiently as in the controls, the ring could be required to reposition the outermost cells and push them towards the otic capsule. These observations bring completely new knowledge on how the perilymphatic space is formed and provides the first molecular regulator, GATA2, for the process.

We also observed that in *Gata2^{cko}* mutants, the number of mesenchymal cells surrounding the superior duct was higher than in controls, while no significant differences occurred in regions surrounding the posterior and lateral ducts between E15.5-18.5. These results may show differences in the clearance mechanism, GATA2 could be required more for superior duct perilymphatic space clearance than for the others. Temporal differences also may exist between the three semicircular ducts to clear their surrounding regions.

Programmed cell death has been suggested as the key mechanism in vestibular and cochlear perilymphatic space clearance (Nikolic et al., 2000; Chang et al., 2002). We, however, detected very little cell death in the inner mesenchyme throughout the development. Thus, the extent of observed cell death does not appear to be sufficient explanation for the reduction in cell numbers detected during perilymphatic region formation. Therefore, we believe that mechanisms other than apoptosis control the mesenchymal cell clearance in the semicircular duct surrounding region. This is strongly supported by the observation that a complete loss of programmed cell death during inner ear development does not cause any

obvious perilymphatic clearance problems in the vestibule or cochlea (Cecconi et al., 2004; unpublished observations).

In the lieu of programmed cell death we considered another possible explanation for the removal of outermost mesenchymal cells from the perilymphatic space. These cells could be pushed towards the condensing capsule and be incorporated into the forming temporal bone. In fact, we noticed that in some areas the perilymphatic and capsule mesenchyme form a continuous tissue with no border or space in between. The incorporation of the perilymphatic mesenchyme into the bone could involve certain adhesion molecules, but nothing is known of the regulation of adhesion in this area. Interestingly, however, we recently observed that *Gata2* expression overlaps with *Gjb2* in the inner mesenchyme of the perilymphatic region, and especially in the ring structure at E16.5-17 (unpublished observations). CONNEXIN 26, encoded by *Gjb2*, may function in cell adhesion and motility, and a role in the maintenance of the endolymph ionic composition has been suggested (Kikuchi et al., 2000; Wei et al., 2004). Interestingly, our microarray analysis showed that *Gjb2* expression was affected in *Gata3*-deficient early inner ear epithelium (Table1 and Fig.4 in II) making it a candidate downstream gene of GATA3. Since GATA2 and GATA3 are able to bind to the same DNA GATA elements, it is tempting to speculate that *Gjb2* could be downstream of GATA2 in the perilymphatic mesenchyme and possibly participate in the clearance process through the formation of the ring-like structure. Whether *Gjb2* is directly regulated by GATA2 (and/or GATA3) remains to be elucidated.

An interesting observation in the *Gata2*^{cko} inner ear phenotype was that the perilymphatic clearance defect occurred concurrently with the epithelial growth arrest. Therefore, the perilymphatic space formation and the semicircular duct growth might be closely linked. No clearance defects have been reported, however, in mouse mutants where similar semicircular duct growth defects have been observed. Several other genes that are expressed in the inner mesenchymal cells during otic development have no effects on the clearance of the perilymphatic space in corresponding mutant mice. Thus, the vestibular perilymphatic clearance defect seems to be specifically related to the absence of GATA2.

4.3.5 Epithelial-mesenchymal interactions during inner ear development (IV)

A close connection between the otic epithelium and surrounding mesenchyme suggests their essential cooperation during inner ear morphogenesis, but the molecular basis remains largely unresolved. The otic epithelium derived FGF-signaling, however, is known to regulate expansion of the surrounding mesenchyme required for the gross development of the inner ear (Pirvola et al., 2004).

Inner ear development is in many ways distinct from other organs involving epithelial-mesenchymal interplay due to an extra “intermediate” cell population (the perilymphatic space mesenchyme) whose role has not been characterized in detail. These cells are present between the epithelium and the capsule mesenchyme during the main morphogenetic phases when the different structures are formed and are only removed later during the stages when the inner ear grows to its final size. An interesting question is that how this presumed epithelial signaling occurs to control capsule form/size when a multicellular layer of inner mesenchyme exists between the two interacting compartments. It is also not known if the epithelial signals diffuse far enough or if the inner mesenchymal cells act as a secondary signaling point or as intermediates between the duct epithelium and otic capsule.

We made an interesting observation about the co-development of the membranous and bony labyrinths when analyzing the control and *Gata2*^{cko} ears. While the diametrical size of the semicircular duct epithelia was strongly diminished (by 30-40%) in *Gata2*^{cko} ears, no corresponding decrease in the diameter of the surrounding perilymphatic space area or the thickness of the bony walls of the canal capsules could be detected. These observations

suggest that the size changes in the epithelial compartment do not necessarily affect the size of the surrounding capsule structures and therefore the co-development is no longer very rigorous, at least at the later stages of otic development when all the different ear structures have formed but are still growing to their final size.

4.4 Analysis of the role of GATA2 during midbrain neurogenesis (V)

In the developing mouse CNS, GATA2 plays an important role in the correct development of cranial motoneurons (Nardelli et al., 1999; Pata et al., 1999) and spinal interneurons (Zhou et al., 2000; Karunaratne et al., 2002). Analyzing GATA2 function in neuronal development has, however, been rather complicated due to the early lethality of *Gata2-/-* embryos at E10.5 (Tsai et al., 1994). To gain more knowledge of the role of GATA2, especially during midbrain neurogenesis, we performed a detailed expression analysis and used the Cre-LoxP conditional mutagenesis approach in mice. To inactivate *Gata2* tissue-specifically, we mated mice with the *Gata2*$^{fl/fl}$ allele with the *En1*^{Cre} mouse line expressed in the midbrain and rhombomere 1 (Kimmel et al., 2000). We also performed ectopic overexpression analyses in chicken embryos.

4.4.1 Conserved expression of *Gata2* in the GABAergic neurons of mouse and chicken midbrain (V)

Despite descriptions of *Gata2* expression in the developing mouse midbrain, the association with a particular neuronal subtype remained unclear (Nardelli et al., 1999; Zhou et al., 2000). We compared the expression of *Gata2* with various neuronal subtype markers, specific for dopaminergic, glutamatergic, GABAergic neurons or motoneurons in the mouse and chicken embryonic midbrain. Comparative expression analysis demonstrated that *Gata2* expression coincided with that of GABAergic neuron specific markers and was excluded from the cells of other neuronal lineages in the midbrain at equivalent stages in mouse (E10.5-12.5) and chicken (HH20-24) (Fig.1 and 5 in V). These results suggest that the timing and pattern of the midbrain GABAergic neuron generation could be conserved between mouse and chicken.

The expression of *Gata3* overlapped with *Gata2* during mouse and chicken midbrain development (Fig.1 and 5 in V). Moreover, *Gata3* expression coincided with GABAergic markers making also GATA3 a reliable marker of GABAergic neurons in the midbrain. In addition, *Gata3* expression was lost in *En1*^{Cre}*;Gata2*$^{fl/fl}$ (hereafter *Gata2*^{cko}) mouse embryos, and the ectopic expression of *Gata2* in chicken midbrain was sufficient to induce *Gata3* expression in the targeted neuroepithelium (Fig.4 and 5 in V). These results indicate that *Gata3* expression is regulated by GATA2 in both the mouse and chicken embryonic midbrain. Similar expression dependence between *Gata2* and *Gata3* has been described earlier in mouse ventral spinal cord precursors (Nardelli et al., 1999; Pata et al., 1999; Zhou et al., 2000) and hindbrain serotonergic neurons (Craven et al., 2004).

4.4.2 Expression of *Gata2* during GABAergic neuron generation in the mouse midbrain (V)

Initially the neural tube is a one cell layer thick neuroepithelium, in which cells are well organized according to the cell cycle progression and differentiation status. As cells become post-mitotic precursors and start differentiating, they leave the ventricular zone and migrate into the outer layer (basal side) of the neural tube, called marginal zone. The proliferative progenitor cells are in the ventricular zone, the innermost cell layer surrounding the lumen (apical side) of the neural tube. The progenitor cells are attached both to the outer surface of the neural tube and to the inner ventricular surface. The position of the nucleus in these neuroepithelial cells correlates with the cell cycle stage. During G1 phase, the cell nucleus

migrates towards the basal side of the ventricular zone, where the DNA replication (S phase) takes place. During G2 phase the nucleus migrates to the apical side of the ventricular zone, where the cell division and cytokinesis occurs (Figure 9).

To understand the possible roles of GATA2 in GABAergic neurogenesis of the mouse midbrain, the expression of *Gata2* mRNA and the presence of GATA2 protein was investigated in mouse midbrain at E10.5-12.5 (Fig.1 and 2 in V). This study confirmed the expression of *Gata2* and its relative *Gata3* in regions where GABAergic neurons are born. The presence of GATA2-positive cells was also compared to bHLH transcription factors ASCL1 and HELT that coexist in GABAergic progenitor cells and cooperatively promote the formation of GABAergic neurons in the midbrain (Miyoshi et al., 2004). All three proteins, GATA2, ASCL1, and HELT were present in the ventral midbrain ventricular zone progenitors at E11.5 (Fig.2 in V). While ASCL1-positive cells were uniformly distributed within the apical and basal side of the ventricular zone, HELT was mostly found at the apical side and GATA2-positive cells at the basal side of the ventricular zone (Figure 9). These differences in the neural tube indicate different proliferation and differentiation status of these cells. The BrdU-analysis confirmed that ASCL1 and HELT were present in proliferating progenitor cells (Miyoshi et al., 2004; V). In contrast, GATA2-positive cells did not incorporate the S-phase marker BrdU, but coexisted with the neuronal differentiation marker HuC/D suggesting that GATA2 is initiated in GABAergic progenitor cells as they become post-mitotic precursors and start differentiating. These results suggest that GATA2 may be required to instruct GABAergic fate during differentiation in the mouse embryonic midbrain.

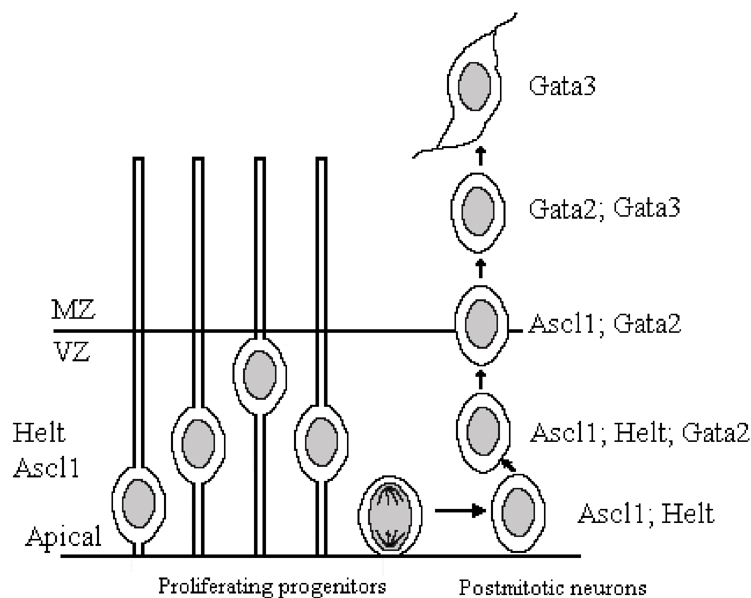

Figure 9. Schematic representation of *Helt*, *Ascl1*, *Gata2* and *Gata3* expression kinetics during GABAergic neurogenesis in the midbrain. Proliferating progenitors in the ventricular zone (VZ) express both *Ascl1* and *Helt*. After neurogenic cell cycle exit, *Ascl1* and *Helt* are expressed in early GABAergic precursor cells together with *Gata2*. During the migration of precursors out of the VZ towards the marginal zone (MZ), *Helt* becomes downregulated and *Gata2* upregulated. In the border of VZ and MZ, GABAergic precursors express *Ascl1* and *Gata2*. *Gata3* becomes expressed in the MZ during the GABAergic neuron differentiation.

4.4.3 GATA2 is essential for GABAergic neuron differentiation in midbrain (V)

To find direct evidence on the role of GATA2 in GABAergic neuron development, we performed loss-of-function experiments in mouse embryos and gain-of-function assays in chicken embryos (V). With these analyses, GATA2 was identified as the first post-mitotic selector gene for the GABAergic over the glutamatergic neuron identity in the midbrain.

4.4.3.1 *Gata2* loss-of-function analysis in mouse midbrain (V)

Nakatani et al. (2007) presented a dorsoventral map of the mouse midbrain at E11.5 that is divided into seven (m1-m7) gene expression domains. At E10.5, the GABAergic neurons rise from domains m3-m5 and, later in development from the dorsal domains m1 and m2. The ventral m6 domain is active in glutamatergic neurogenesis. The gene expression analysis in our study further refined the map of Nakatani et al. (2007) so that midbrain domain m4 gives rise to both GABAergic (dorsal part) and glutamatergic (ventral part) neurons (Fig.8 in V).

We abolished *Gata2* expression in the mouse midbrain around E8-8.5 using the *En1*^{Cre} mouse line (Kimmel et al., 2000) crossed with the *Gata2*^{flox} allele containing mice. Changes in embryonic brain morphology and ventricular zone patterning were not detected in the resulting *Gata2*^{cko} mutants. The mRNA expression analyses showed that GABAergic neuron markers (*Gad1*, *Gad2,* and *Gata3*) were completely lost in *Gata2*^{cko} midbrain at E11.5 and E13.5 indicating no GABAergic neuron precursor formation (Fig.4 in V). The detailed analyses of the number of caspase3-positive nuclei, phospho-histone H3-positive mitotic nuclei and BrdU-incorporating S-phase nuclei in the GABAergic progenitor cell layer showed no clear changes in cell survival or proliferation in *Gata2*^{cko} embryos compared to controls. In addition, the expression of a cell cycle inhibitor *p57* (*Cdkn1c*) was unchanged in *Gata2*-deficient progenitor cells showing that cells are able to exit the cell cycle and become post-mitotic even without *Gata2*. The GABAergic progenitor cell layer in the midbrain showed normal appearance in *Gata2*^{cko} mutants.

Further analysis in *Gata2*^{cko} embryos demonstrated that expression of post-mitotic glutamatergic markers (*Slc17a6* and *Pou4f1*) was either expanded to the marginal zone or was ectopically found in the original GABAergic m3 domain, suggesting that *Gata2*-mutant cells seemed to specifically acquire the expression of genes characteristic for glutamatergic precursor subtypes. For instance, in the absence of *Gata2*, the cells in the m5 midbrain domain did not express the GABAergic marker (*Lhx1*), but instead started to express glutamatergic marker (*Nkx6*-1) similar to their adjacent glutamatergic m6 domain (Fig.4 in V). The m4 that gives rise to both GABAergic and glutamatergic neurons in controls, also became a solely glutamatergic marker-expressing domain in *Gata2*^{cko} midbrains. These results suggest that in the absence of *Gata2* all post-mitotic precursor cells have undergone a fate transformation and adopted the excitatory glutamatergic neuron fate instead of the inhibitory GABAergic phenotype. Taken together, inactivation of *Gata2* does not affect GABAergic progenitor cell proliferation, survival, or neurogenic cell cycle exit, but it is required for the neuronal subtype commitment in the early stages of precursor differentiation.

4.4.3.2 *Gata2* gain-of-function analysis in chicken midbrain (V)

Earlier studies show that the ectopic expression of *Gata2* is able to induce an interneuron phenotype in the spinal cord (Karunaratne et al., 2002) and serotonergic neuron fate in the hindbrain (Craven et al., 2004) at the expense of the adjacent cell fates. We were interested in finding out what happens when *Gata2* cDNA is introduced ectopically into the chicken dorsal midbrain prior to GABAergic differentiation. We were especially interested whether GATA2 would be sufficient to switch on the GABAergic differentiation pathway in the midbrain.

As mentioned previously, *Gata2* expression is conserved in chicken and mouse midbrain development. In both species, the expression is first initiated in the ventral midbrain (around

E10.5 in mouse and HH20 in chicken) that subsequently becomes to broaden towards the dorsal part (E12.5 and HH24). The expression of several genes characteristic for GABAergic neurons showed that GABAergic development seems to occur in a similar fashion in chicken and mouse (Fig.5 in V). Due to the better survival of chicken embryos in gain-of-function assays, we used the chicken model to study the effects of *Gata2* overexpression to the neurogenesis in the midbrain. The *Gata2* expression vector was delivered by *in ovo* electroporation into HH14-16-stage chicken dorsal midbrain that later gives rise to both glutamatergic and GABAergic neurons. After 24 or 48 hours, we detected abundant expression of GABAergic markers (*Lhx1*, *Gata3*, and *Gad1*) in the targeted dorsal midbrain regions, and at the same time, the glutamatergic marker *Ngn2* expression was reduced suggesting a specific induction of GABAergic differentiation in the expense of neighboring glutamatergic cells (Fig.5 in V).

In conclusion, the GATA2 gain-of-function analysis demonstrated that GATA2 is sufficient to induce a GABAergic phenotype in chicken midbrain neuroepithelium.

4.4.4 GATA2 is dispensable for GABAergic neurogenesis in rhombomere 1 (V)

The expression of *Gata2* was detected in the domains of GABAergic neuron production in the rhombomere 1. In *Gata2*^{cko} mouse embryos, the expression of *Gata2* was also lost in rhombomere 1 providing new data about the role of GATA2 in this anterior segment of the hindbrain. Interestingly, the expression of GABAergic markers (*Gad1* and *Gata3*) appeared unaffected in the *Gata2*^{cko} rhombomere 1 (Fig.6 in V). In contrast to the essential role of GATA2 in midbrain GABAergic neurogenesis, GATA2 seems to be dispensable for that matter in anterior hindbrain. Thus, diverse mechanisms seem to regulate the formation of GABAergic neurons in different brain compartments.

Using the *in vitro* cultures of the mutant tissue, GATA2 was identified as an essential serotonergic neuron fate determinant in the rhombomere 1 (Craven et al., 2004). The *Gata2* conditional mutants represent a useful tool for analyzing GATA2 function in the developing embryo. The mRNA and protein expression analysis showed that serotonergic markers (*Lmx1b*, *Pet1*, and *5-HT*) were completely absent in *Gata2*^{cko} rhombomere 1 region. In addition, the *Gata3* expression was absent in *Gata2*-deficient rhombomere 1 serotonergic compartment, whereas it was unaffected in GABAergic neurons. These results show that GATA2 is required for serotonergic neuron development in the rhombomere 1, and that GATA2 regulates the expression of *Gata3* in these cells.

4.5 Comparison of the roles of GATA2 and GATA3 in inner ear and CNS (II, III, IV, V)

We showed that GATA2 regulates neuronal cell fate specification in the developing midbrain (V). Similarly, GATA2 is required for the specification of serotonergic neurons in the rhombomere 1 of the hindbrain (Craven et al., 2004) and ventral interneurons in the spinal cord (V; Zhou et al., 2000; Peng et al., 2007). Thus, GATA2 appears to have a conserved function in different parts of the CNS. The function of GATA2 in neuronal cell fate specification has been suggested to involve a direct activation of cell type specific genes, such as *Pet-1* that is a serotonergic fate determinant in the hindbrain (Hendricks et al., 2003). The promoter region of *Pet-1* includes two conserved GATA binding sites, and direct binding of GATA2 to these elements is essential for *Pet-1* expression (Krueger and Deneris, 2008). In addition to direct regulation of cell-type specific genes, GATA2 can also drive neuronal progenitor cells out of the cell cycle and into differentiation pathway in the chicken spinal cord (El Wakil et al., 2006). This study suggested that GATA2 interferes with the regulation of cell cycle components, such as CyclinD1 and CDKN1B, and represses NOTCH pathway to

negatively control proliferation of the neuronal progenitor cells (El Wakil et al., 2006). Our results demonstrated, however, that in mouse midbrain GATA2 has no direct cell cycle regulatory function (V). Thus, GATA2 appears to control at least two different events during neuronal differentiation in vertebrate CNS depending on the cell type.

Our results regarding inner ear development showed that GATA2 positively controls cell proliferation in the nonsensory epithelium and contributes to the diametrical growth of the three semicircular ducts. Additionally, GATA2 is particularly important for the formation of the mesenchymal-free vestibular perilymphatic space between the membranous and bony labyrinth (IV). Although, the mechanisms regulating perilymphatic space formation are not well understood, our data suggested that GATA2 contributes to the creation of a mesenchymal ring-like structure that seems to be important for the clearance of the perilymphatic space (IV). The formation of this ring most likely involves yet unknown adhesion activities. Thus, in contrast to the brain, GATA2 is not involved in cell fate determination during inner ear development, but seems to have a main role in promoting cell proliferation in the epithelium and possibly adhesion in the mesenchyme. The target genes and binding partners of GATA2 in inner ear are currently unknown and remain to be elucidated.

Similar to GATA2, GATA3 has also an essential role in neuronal development. For instance, GATA3 is specifically required for the differentiation of serotonergic neurons in the caudal Raphe nuclei (van Doorninck et al., 1999) and for neuronal migration (Pata et al., 1999). According to our results *Gata3* expression is largely overlapping with *Gata2* in the GABAergic precursors in midbrain (V). However, *Gata3* is expressed later than *Gata2* and can be detected only in post-mitotic neurons of the marginal zone (Figure 9) suggesting that it may have a slightly later role in neuronal differentiation compared to GATA2, which regulates specification.

In inner ear, GATA3 is also essential for the development of cochlear ganglion neurons, although its function seems to be different in mouse and chicken. In mouse, *Gata3* is expressed throughout the cochlear ganglion development and therefore may regulate multiple aspects in otic neurogenesis (Lawoko-Kerali et al., 2004; Lilleväli et al., 2004). Indeed, GATA3 is known to be important for cochlear afferent neuron specification, as well as the pathfinding of efferent neurons to the inner ear sensory epithelia (Karis et al., 2001; Lawoko-Kerali et al., 2004; Jones and Warchol, 2009; Duncan et al., 2011). On the contrary, in chicken GATA3 appears to be significant after terminal mitosis, during the differentiation of cochlear neurons (Jones and Warchol, 2009; I). Besides the essential role in otic ganglion and CNS neurogenesis, our data showed that GATA3 is specifically required for the differentiation of cochlear sensory epithelium (III) suggesting that GATA3 may have similar functions in both developing neurons and hair cells. In contrast to the sensorineural tissue, we could not find clear indications of a role for GATA3 in cell differentiation in the inner ear nonsensory epithelium. Instead, there GATA3 may control epithelial adhesion and therefore influence cell motility and intraepithelial migration during otic vesicle formation (II). Our microarray analysis identified several potential GATA3 target genes that encode for proteins with adhesion properties (II).

Similar to GATA2, a potential function of GATA3 in cell cycle regulation has been reported in a variety of different tissues. For example, GATA3 controls proliferation of mesonephric cells (Grote et al., 2006), as well as mammary epithelial cells (Kouros-Mehr et al., 2006) and lens fiber cells (Maeda et al., 2009). Moreover, the GATA3 conditional deletion in hair follicles indicated that the expression of multiple cell cycle regulatory genes was altered (Kurek et al., 2007). Similarly, in cochlear sensory epithelium GATA3 is required for the upregulation of *Cdkn1b* expression and thus its function could be closely linked to cell

cycle arrest and initiation of differentiation. How GATA3 functionally coordinates cell cycle regulation with differentiation in a variety of tissues requires, however, additional studies.

Taken together, the two GATA factors have important regulatory roles during inner ear and CNS development related to cell cycle progression and/or the closely linked initiation of differentiation. In addition, both factors appear to control cell adhesion in very specific events during inner ear morphogenesis involving either the epithelium or the adjacent mesenchyme. The contribution of GATA2 and GATA3 in inner ear and CNS development is summarised in Table 6.

Table 6. Involvement of GATA2 and GATA3 in inner ear and CNS.

Factor / Tissue	GATA2	GATA3
Inner ear	Diametrical growth of the semicircular ducts by positive regulation of epithelial cell proliferation and clearance of the vestibular perilymphatic space from mesenchymal cells (IV)	Invagination of the otic placode (II). Differentiation of the cochlear sensory epithelium (III; Duncan et al., 2011). Specification of cochlear afferent neurons and pathfinding of efferent neurons to the otic sensory epithelia (Karis et al., 2001; Lawoko-Kerali et al., 2004; Duncan et al., 2011)
CNS	Differentiation of GABAergic neurons in the midbrain (V), serotonergic neurons in the hindbrain (Craven et al., 2004) and ventral interneurons in the spinal cord (Zhou et al., 2000; Peng et al., 2007). Negative regulation of progenitor cell proliferation in the spinal cord (El Wakil et al., 2006).	Migration of facial brachiomotor neurons to rhombomere 6 (Pata et al., 1999). Differentiation of serotonergic neurons in the caudal Raphe nuclei (van Doorninck et al., 1999).

Concluding remarks

The morphogenetic development of the inner ear and CNS is a complex multistep process, of which molecular and cellular details are still largely unclear. The progress in molecular biology and genetics methods in the past decades, however, has significantly advanced the understanding of mammalian inner ear and CNS formation. In this study, conventional gene targeting as well as conditional genetic manipulation of mouse embryos was combined in expression analyses and gain-of-function approaches in chicken embryos to clarify the role of GATA factors in inner ear and CNS development. This study serves as a basis for the elucidation of the molecular hierarchies involving GATA factors in both tissues. Also, new data of several ill-understood developmental phenomena were collected, such as the early morphogenesis of the otic placode, inner ear sensory fate establishment and differentiation, formation of the perilymphatic space surrounding the semicircular ducts, and the development of midbrain GABAergic neurons.

We identified GATA3 as the first key factor controlling otic placode invagination most likely through the regulation of intrinsic epithelial properties, such as adhesion. More recently, by inactivation of another transcription factor gene, *Sox9*, the importance of proper adhesion between the placodal cells has been confirmed as one or maybe the most crucial aspect during invagination (Barrionuevo et al., 2008). Moreover, both studies suggested that the extrinsic influence from the adjacent hindbrain is not necessary for the otic cup and vesicle formation in mouse embryos. Interestingly, both GATA3 and SOX9 seem to operate at least in part by influencing *Epha4* expression in the otic epithelium, GATA3 negatively in the vesicle, and SOX9 positively in the placode. This remains to be verified, whether GATA3 and SOX9 directly regulate *Epha4* expression and especially, does GATA3 also control *Epha4* in the placode stage.

This study included a large-scale expression analysis that identified a catalog of genes potentially involved in early inner ear development downstream of *Gata3*. At the beginning of this study, GATA3 was known to have a role during otic vesicle formation (Karis et al., 2001) and thus, the analysis was performed at otic vesicle stage. We, however, revealed that inner ear development was already severely affected at the vesicle stage. Thus, it would be interesting to perform a similar analysis at the placode stage, closer to the initiation of the *Gata3-/-* phenotype to see if additional downstream genes could be identified.

The pharmacologically-rescued *Gata3-/-* embryos provide a good opportunity to follow the inner ear development after E11.5 and especially to verify the sensory epithelium differentiation in the absence of *Gata3*. We showed that the initial sensory fate determination occurred in *Gata3-/-* ears and that in the vestibule hair cells are forming. GATA3, however, was specifically required to generate hair cells in the cochlea. In mouse and humans, haploinsufficiency of *Gata3* leads to deafness due to failure in cochlear sensory cell maintenance (van der Wees et al., 2004). Since *Gata3* is expressed in cochlear hair cells during embryogenesis and adulthood, it could function cell-autonomously in sensory cell maintenance. *Gata3* is, however, also expressed in the endolymph-secreting tissue, the mammalian stria vascularis. Thus, it is still unclear, whether GATA3 influences hair cell maintenance directly due to its presence in hair cells or indirectly from the endolymph secreting cells, or both. To gain more information, it would be important to generate a *Gata3* conditional allele, which would allow study the GATA3 role at later stages in cochlear hair cell development and maintenance without the early morphogenetic defects. These mice would also enable the search of GATA3 target genes in cochlear sensory epithelium. Moreover, it would be interesting to find out whether GATA3 influences vestibular and cochlear morphogenesis and/or cell differentiation from the adjacent mesenchyme where it is strongly expressed.

The inner ear is a fairly late-developing structure in the mouse and therefore some questions may be unanswerable by basic loss-of-function studies due to the early embryonic lethality. On these occasions, the importance of creating conditional mutants increases. Here, the conditional mutagenesis of *Gata2* was carried out since the conventional inactivation did not lead to any defects in inner ear development. New aspects emerged concerning the growth of epithelial semicircular ducts and the clearance of the perilymphatic space between the membranous and bony labyrinth. This study described GATA2 as the first factor known to be required for the mesenchymal cell clearance and demonstrated that programmed cell death is not the main mechanism behind the perilymphatic space formation, in contrast to the previous hypotheses. The exact process of clearance, however, still remains unclear despite cell adhesion properties most likely having an important role. To clarify to what extent GATA2 regulates otic morphogenesis through its expression in the otic epithelium and, on the other hand, through the mesenchymal expression additional Cre-mouse lines should be used in the future.

The midbrain GABAergic neurons are associated with many behavioral functions and psychiatric diseases. Therefore, the understanding of GABAergic neuron development in the midbrain is particularly important to finding treatments for serious diseases. This work produced novel data about GATA2 and showed that it is both necessary and sufficient to induce GABAergic neuron differentiation in the midbrain. Future studies should focus on finding GATA2 target genes that could elucidate the GATA2-regulated mechanisms in GABAergic fate specification and provide information on novel genes and pathways involved in the process. Additionally, GABAergic neurons in the auditory cortex express *Gata2*, thus it would be also interesting to study the function of GATA2 in brain regions mediating hearing processes.

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