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MOLECULAR REGULATION OF THE DEVELOPMENT AND DEATH OF INNER EAR HAIR CELLS AND NEURONS

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Academic dissertation

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CONTENTS

ABSTRACT

The inner ear originates from an ectodermal thickening, the otic placode, which invaginates and closes to a vesicle. Further morphogenesis leads to the formation of the complex labyrinth-like structure of the inner ear. The otic placode gives rise to the hearing (cochlea) and balance (vestibular) organs, comprising the sensory hair cells, and to the afferent sensory neurons of the cochlear and vestibular ganglia. In mammals, inner ear sensory cells undergo terminal mitosis during embryogenesis. Therefore, during postnatal life, trauma-induced sensory cell loss is an accumulating phenomenon leading to permanent hearing impairment. This is because these cells do not regenerate. We have studied the molecular mechanisms regulating sensory cell formation during embryogenesis and death in the traumatized adult inner ear. This knowledge might help to design strategies to induce therapeutically hair cell regeneration in adult mammals and to prevent trauma-induced sensory cell death.

We found that signaling by two neurotrophins, neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), through their tyrosine kinase TrkC and TrkB, receptors, respectively, is essential for the establishment of afferent innervation of the inner ear. *Bdnf* and *Nt-3* mRNAs are expressed in the inner ear sensory epithelia, which comprise the peripheral target fields of the cochlear and vestibular sensory neurons. *In vitro*, NT-3 and BDNF promoted survival and differentiation of embryonic inner ear neurons. These results have been later verified by others by the use of transgenic technology. The inner ear serves as an excellent example of the neurotrophic hypothesis stating that the innervating neurons of the peripheral nervous

system are dependent on their peripheral targets. In adults, trauma-induced loss of auditory hair cells leads to progressive degeneration of the innervating cochlear neurons. This neuronal degeneration can be prevented by the delivery of exogenous neurotrophic factors into the cochlea.

In contrast to mammals, birds have retained the plasticity for hair cell regeneration. Formation of new hair cells in adult chicks is followed by reinnervation and functional recovery. The changes that we found in the expression of *Bdnf* and the *TrkB* receptor isoforms during regeneration suggest that this signaling regulates the reinnervation process, and that it recapitulates many aspects of embryonic innervation.

Our expression studies suggested that signaling by fibroblast growth factor 10 (FGF10) and the IIIb isoform of FGF receptor 2 (FGFR2IIIb) might have an impact on inner ear development. By analyzing the ear phenotype of *Fgfr2(IIIb)* null mutant mice, we found that FGF/FGFR2(IIIb) signaling blocks early inner ear morphogenesis. The mutation leads to the formation of a rudimentary inner ear, which lacks all sensory structures. In contrast to these global inner ear defects, we found that signaling by FGFR1 specifically acts on the pool of early precursor cells giving rise to the cochlear hair cells and supporting cells. In the conditionally inactivated *Fgfr1* mutant mice, in which *Fgfr1* inactivation is targeted to the otic epithelium, the majority of cochlear hair cells fail to develop. This knowledge might be used to design strategies for activation and/or expansion of the precursor cell pool that could give rise to new hair cells, even in the adult hearing organ.

Hair cells have been shown to die through apoptosis following inner ear-specific traumas such as exposure to noise and ototoxic drugs. We found that the c-Jun N-terminal (JNK) cascade – associated with injury and apoptosis – is activated in stressed hair cells. CEP-1347 is a derivative of the indolocarbazole K252a, which specifically prevents activation of the JNK cascade. We found that neomycin-induced hair cell death was near-totally prevented in cochlear *in vitro* cultures when CEP-1347 was added to the medium. *In vivo*, systemic delivery of CEP-1347 to noise- or gentamicin-exposed guinea pigs attenuated loss of cochlear and vestibular hair cells and decreased auditory threshold shift as compared to non-treated (only noise- or gentamicin-exposed) guinea pigs. These results suggest that JNK inhibitors such as CEP-1347 may offer therapeutic potential in the traumatized inner ear by preventing sensory cell death.

ORIGINAL PUBLICATIONS

This thesis is based on the following articles and a manuscript, which are referred to in the text by their Roman numerals:

I Pirvola U, Ylikoski J, Palgi J, Lehtonen E, Arumäe U, Saarma M. (1992) Brain-derived neurotrophic factor and neurotrophin 3 mRNAs in the peripheral target fields of developing inner ear ganglia. Proc Natl Acad Sci USA 15:9915-9919.

II Pirvola U, Arumäe U, Moshnyakov M, Palgi J, Saarma M, Ylikoski J. (1994) Coordinated expression and function of neurotrophins and their receptors in the rat inner ear during target innervation. Hear Res 75:131-144.

III Pirvola U, Hallböök F, Xing-Qun L, Virkkala J, Saarma M, Ylikoski J. (1997) Expression of neurotrophins and Trk receptors in the developing, adult, and regenerating avian cochlea. J Neurobiol 33:1019-1033.

IV Pirvola U, Xing-Qun L, Virkkala J, Saarma M, Murakata C, Camoratto AM, Walton KM, Ylikoski J. (2000) Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J Neurosci 20:43-50.

V Ylikoski J, Qing-Qun L, Virkkala J, Pirvola U. (2002) Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair-cell death. Hear Res 163:71-81.

VI Pirvola U, Spencer-Dene B, Xing-Qun L, Kettunen P, Thesleff I, Fritzsch B, Dickson C, Ylikoski J. (2000) FGF/FGFR-2(IIIb) signaling is essential for inner ear morphogenesis. J Neurosci 20:6125-6134.

VII Pirvola U, Ylikoski J, Trokovic R, Hébert J, McConnell S, Partanen J. FGFR1 is required for the development of the auditory sensory epithelium. Neuron, in press.

In addition, some unpublished results and data from our other papers are presented.

ABBREVIATIONS

ABR	auditory brainstem response
ASK1	apoptosis signal-regulating kinase 1
BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
bp	base pair
BrdU	5′-bromo-2′-deoxyuridine
cDNA	complementary deoxyribonucleic acid
DAB	3′-3′-diaminobenzidine
dB	decibel
DNA	deoxyribonucleic acid
DIG	digoxigenin
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
Foxg1	forkhead box G1
GA	glutaraldehyde
GCK	germinal center kinase
GDNF	glial cell line-derived neurotrophic factor
GER	greater epithelial ridge
HC	hair cell
Hes	hairy and enhancer of split homolog
HPK-1	hematopoietic progenitor kinase
HSPG	heparan sulfate proteoglycan
IHC	inner hair cell
Ig	immunoglobulin
JNK	c-Jun N-terminal kinase
KHS	kinase homologous to SPS1/STE20

LER	lesser epithelial ridge
MAPK	mitogen-activated protein kinase
MEKK	mitogen-activated protein kinase kinase kinase
MKK	mitogen-activated protein kinase kinase
MLK	mixed-lineage kinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
Ngn	neurogenin
NIK	NF-kappaB inducing kinase
NT	neurotrophin
NT-3	neurotrophin-3
NT-4	neurotrophin-4
OHC	outer hair cell
PAK	p21-activated kinase
PFA	paraformaldehyde
PNS	peripheral nervous system
PCR	polymerase chain reaction
ROS	reactive oxygen species
SPL	sound pressure level
Tpl-2	Tachypleus plasma lectin-2
Trk	tropomyosin receptor kinase
TUNEL	terminal deoxynucleotidyl transferase-mediated UTP nick end labeling
UTP	uridine triphosphate
UV	ultraviolet
WNT	vertebrate homologue of *Drosophila* wingless gene

INTRODUCTION

Significant hearing loss compromising communication occurs in about 15% of the population and more than one-third of those who are over 65 years old. Hearing loss can be due to environmental or genetic factors, but is usually a combination of both. In the case of environmental factors, hearing impairment most often results from the loss of auditory hair cells (HCs). We are born with a complement of about 16000 HCs and 30000 auditory neurons in each ear. HCs and neurons of the mammalian inner ear do not regenerate during postnatal life. Therefore, loss of each sensory cell, e.g. due to noise, infections or toxic drugs, is irreversible and cumulative. If enough HCs and/or neurons are lost, the end result is deafness or permanent balance dysfunction. Currently there is no effective treatment for these conditions in humans. However, much progress has been made during recent years in elucidating the molecular mechanisms involved in development and death of HCs and in understanding the requirements of inner ear neurons for HC-derived trophic factors. Therefore, there are reasons for optimism that a therapy for healing hearing and balance disorders will be developed in the future.

This work focuses on the sensory cells of the inner ear, i.e. the HCs of the cochlea and vestibular organs, and the afferent neurons of the cochlear (spiral) and vestibular ganglia. Hair cells of the auditory sensory epithelium, the organ of Corti, sense sound vibrations. Hair cells of the saccular and utricular sensory epithelia (maculae) and HCs of the three ampullary sensory epithelia (cristae) sense gravity changes as well

as linear and angular acceleration. In addition to HCs, the nearby supporting cells – although their non-informative name- are structurally and functionally essential components of the inner ear sensory epithelia. Hair cells are the peripheral targets not only for afferent innervation, but also for efferent innervation originating from brainstem nuclei.

This work deals with the following central questions within the inner ear field: (1) What are the molecular mechanisms that regulate morphogenesis of the inner ear, the complex structure, which originates from a simple ectodermal thickening, the otic placode? (2) What are the signaling mechanisms that, during embryogenesis, promote proliferation and differentiation of the precursor cells giving rise to the inner ear sensory epithelia, thus leading to the correct pattern and proportions of HCs and supporting cells? It is possible that knowledge of the mechanisms regulating HC generation during embryogenesis could be exploited in designing therapies aimed at stimulating HC regeneration in adult mammals in which HC replacement does not normally occur. (3) What are the molecular mechanisms of sensory cell death in the traumatized adult inner ear? Also this knowledge could possibly be applied in a future therapy, the aim being the attenuation or blockade of trauma-induced sensory cell death. (4) What is the nature and source of growth factors that promote survival and differentiation of developing inner ear neurons and that provide neuroprotection in the traumatized adult inner ear? Functionally and anatomically the human inner ear is similar to that of other mammals. Thus, rodents can be used as models when studying the underlying mechanisms of ear function. Many of these mechanisms can be also studied in non-mammalian species. However, one distinct feature, which

separates mammals from fishes, reptiles and birds is that the latter species have retained the plasticity for HC renewal. Knowledge of the mechanisms regulating HC regeneration in for example birds might help to understand whether it is possible to induce the same process by exogenous means in mammals.

REVIEW OF THE LITERATURE

I Inner ear development

The vertebrate inner ear arises from the otic placode, which is formed close to the hindbrain rhombomeres 5 and 6. The placode invaginates and closes to form a vesicle, also known as the otocyst (Fig. 1A). Morphogenesis leads to the formation of thickened sensory patches, which give rise to the cochlea and vestibular organs. Neuronal precursors start to delaminate from these thickened regions within the otic epithelium and migrate to form the cochlear and vestibular ganglia. At the same stages, the non-sensory appendages, the endolymphatic and semicircular ducts, start to bud out from the vesicle. A little later, the cochlear duct starts to extend from the otocyst. Thus, the cochlea and vestibular organs (Fig. 1B), the neurons of the cochlear and vestibular ganglia, and the secretory and simple epithelia lining the fluid-filled cavities of the inner ear labyrinth are all derived from the otic placode. These soft compartments are surrounded by the periotic mesenchyme, which chondrifies and forms the otic capsule. Neural crest contributes to the melanocytes of stria vascularis and Schwann cells of the inner ear ganglia and to the autonomic innervation of the inner ear, the fibers of which originate from the superior cervical ganglion.

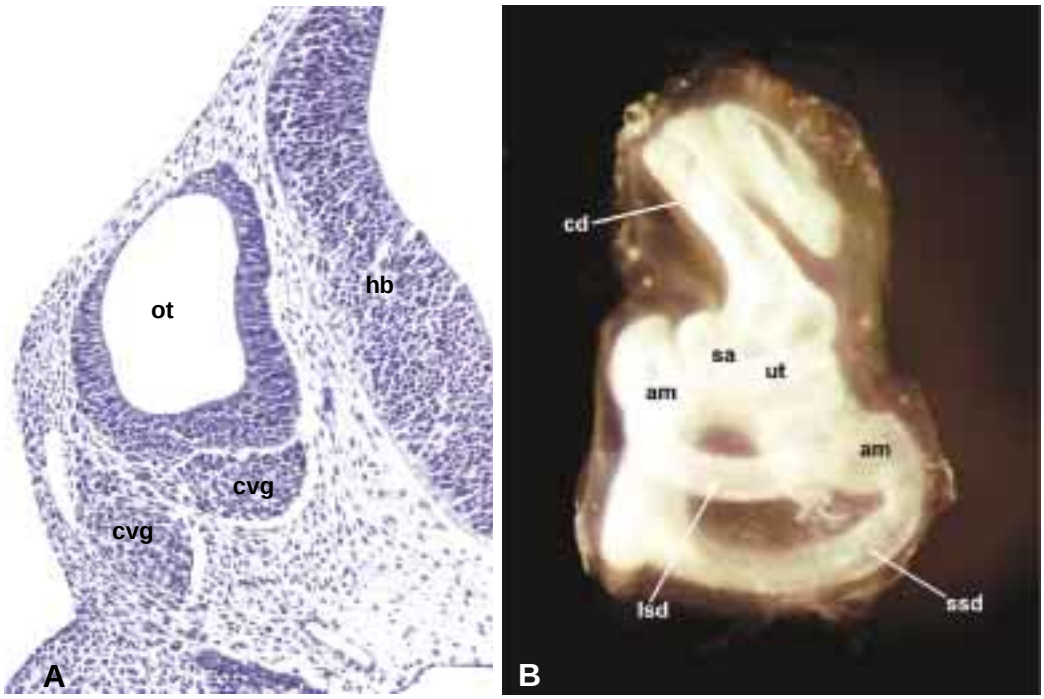

Figure 1. Mouse inner ear at embryonic day 10 (A) and embryonic day 18 (B). (**A**) Hematoxylin-stained transverse section through the otocyst (ot). One of the first signs of morphogenesis, delamination of neuronal precursors from the otic epithelium and their migration to form the cochleovestibular ganglion (cvg) is seen. hb, hindbrain.(**B**) Latex paint solution has been microinjected into the cochleovestibular membraneous labyrinth at birth to illustrate the global structure. Cd, cochlear duct; ssd, superior semicircular duct; lsd, lateral semicircular duct. Approximate locations of sacculus (sa), utriculus (ut) and two ampullae (am) are also shown.

1. Induction of the otic placode

A long-lasting goal has been to identify the tissue source and molecular nature of signals inducing otic placode formation. Primary induction has not been

directly studied in the present thesis, but will be discussed here since it precedes the early morphogenesis experimentally addressed by us. Classic transplantation studies indicate that the formation of the otic placode is induced by signal(s) from the hindbrain (Stone, 1931; Waddington, 1937; Harrison, 1935; Yntema, 1933). Some early studies also suggested that mesoderm plays a role in otic induction (Jacobson, 1963). Recent data using molecular markers suggest that otic induction occurs earlier than had been previously thought based on morphological criteria (Groves and Bronner-Fraser, 2000). These new data also show that placodal induction proceeds in successive steps and since these steps can be experimentally separated from each other, they are likely to be regulated by different molecular signals. Despite extensive speculation, the molecular basis of otic induction has not yet been elucidated. Importantly, molecular regulation of otic induction seem to differ considerably between different species. It was recently suggested that mesoderm-derived fibroblast growth factor 19 (FGF19) followed by hindbrain-derived WNT are required for otic induction in the chick (Ladher et al., 2000), but direct genetic evidence of the role of these factors is still lacking. Several other lines of evidence point also to the involvement, either direct or indirect, of FGFs in otic induction (Vendrell et al., 2000; Phillips et al., 2001).

2. Regulation of early inner ear morphogenesis

Morphogenesis is responsible for the correct spatial patterning of cell populations – giving rise to the three-dimensional organization of the different organs of the body. It is coordinated at the cellular level with proliferation, death, cell fate determination

and differentiation (reviewed by Hogan, 1999). There is continuous debate about the impact of surrounding tissues on early morphogenesis of the otic epithelium. Classic microsurgical experiments have shown that, in addition to the otic placode induction, the periotic mesenchyme and the hindbrain are important for the development of the otic vesicle before its closure. Early data have suggested that the hindbrain guides axial (anterior-posterior) polarity of the vesicle. More recent experiments support the role of hindbrain-derived signals in patterning of the inner ear. Specifically, inactivation of genes such as *Hoxa1* and *kreisler*, which are expressed in the hindbrain and which control hindbrain segmentation and rhombomere identity, lead indirectly to dysmorphogenesis of the early inner ear of the mouse (Lufkin et al., 1991; Chisaka et al., 1992). In addition to the stages before the vesicle closure, mesenchyme appears to have an impact on the later development of the otocyst. Grafting experiments in birds indicate that global patterning of the otocyst is perturbed if the surrounding otic mesenchyme is removed (Swanson et al., 1990). Grafted otocysts surrrounded by a foreign mesenchyme showed no outgrowth of non-sensory (semicircular canals) or sensory (cochlea) appendages. The sensory epithelium developed in these grafts, but instead of the eight sensory patches (the future sensory organs) that develop in normal avian ears, the grafts contained only a few large sensory patches. Importantly, normal local arrangement of HCs and supporting cells was found in the sensory patches of the grafted otocysts. Thus, the otic mesenchyme seems to be required for global shaping of the otocyst and perhaps also for proper positioning of the sensory patches within the otic epithelium, but not for cellular development within the patches. A drawback of these grafting

experiments is that they were performed after the otocyst closure. Thus, they do not exclude the possibility that earlier mesenchyme-to-epithelium interactions might be involved for example in the initiation of the development of sensory patches.

More recent genetic evidence indicate that otic epithelium-autonomous signaling contributes to otocyst morphogenesis. *Fgf10* is expressed in the sensory patches and its receptor, the *IIIb* isoform of *Fgf receptor 2*, *Fgfr2(IIIb*), in the future non-sensory regions of the otocyst epithelium. When *Fgfr2(IIIb)* or *Fgf10* genes are inactivated, the outgrowth of appendages from the vesicle is blocked (paper VI; Pauley et al., 2001) Together, available data suggest that both epithelium- and surrounding tissue-derived signals are required for early inner ear morphogenesis.

The boundary hypothesis established by Meinhardt (1983a,b) has been suggested to explain inner ear patterning (Fekete, 1996) in a similar manner to the patterning of other cell populations of multicellular organisms. The model suggests that gene expression domains segregate the early otic epithelium into lineage-restricted compartments. Transcription factors such as *Otx1* and *Otx2* (Morsli et al., 1999) have been suggested as candidates for dividing the otic epithelium into compartments. Depending on the combination of these selector genes at a particular position, the location and identity of structures such as the future sensory organs, endolymphatic and semicircular ducts, and the origin of neuronal precursors might be specified at the boundaries of interacting genes. In the second step, the selector genes direct the expression of short-range growth

factor(s), which are expressed on one side of a compartment boundary. These factors can act on the receptor-bearing cells in the adjacent compartment. In the third step, these responding cells start to produce long-range organizing molecules (morphogens), which induce expression of target genes in a concentration-dependent manner, controlling growth and patterning of both compartments. Candidate signaling mechanisms directing otocyst patterning according to the boundary model include FGF signaling, which has been suggested to operate between the sensory and non-sensory domains of the otocyst (paper VI). However, although the boundary model is attractive, future experimentation is needed to test its validity.

In addition to growth and transcription factors, extracellular matrix (ECM) molecules, cell-matrix interactions and cell-cell adhesion molecules are likely to exert important roles on both the global and fine-grained development of the inner ear. However, only little direct experimental evidence is currently available. There is one good example showing how a non-collagenous ECM component can regulate the shaping of the ear labyrinth, namely hyaluronan, which controls the formation of the three semicircular ducts (Haddon and Lewis, 1991). The otic vesicle gives rise to three flattened outpockets. The centres of the two opposite faces of each outpocket form axial protrusions into the lumen (Fig. 2). These protrusions eventually contact each other and the opposed epithelial sheets fuse and reorganize, resulting in the formation of a new semicircular duct. Hyaluronan is produced by the epithelial cells forming the axial protrusions. It accumulates between the epithelium and mesenchyme, forming a cell-free space, which allows the epithelium to move and protude into lumen of the

vesicle. When hyaluronan is enzymatically destroyed, the axial protrusion collapses and the corresponding semicircular duct fails to form. In a parallel manner as suggested in the developing inner ear, hyaluronan seems to regulate morphogenesis of organs such as the heart, which also consists of several separate cavities (Haddon and Lewis, 1991).

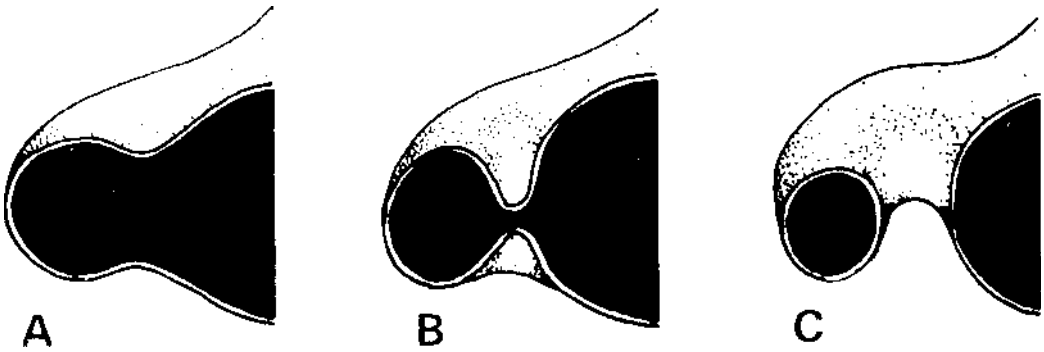

Figure 2. Schematic representation of the three stages of the formation of a semicircular duct. (**A**) Initial outpocketing from the otic epithelium. (**B**) Axial protrusions grow towards the lumen. (**C**) Axial protrusions fuse and create a semicircular duct. From Haddon and Lewis (1991) by permission of the Company of Biologists Limited, Cambridge, UK.

3. Generation of the cochlea

Early morphological studies in non-mammalian species have suggested that the inner ear sensory organs arise from a single ventromedial region of the otocyst (Knowlton, 1967). More recent data using the mouse as a model system and molecular markers, which allow the identification of sensory regions before they can be morphologically (thickened patches) distinguished, challenge some of these earlier data. Based on

gene expression patterns, it has been suggested that the superior and lateral ampullary cristae of the mouse are generated first and that they share a common origin in the anterior part of the otocyst. The cochlea and the maculae of sacculus and utriculus are thought to arise from a single region in the ventral portion of the otocyst and they are separated later in development (Morsli et al., 1998). However, molecular markers neither provide direct evidence of the origin of the different inner ear sensory organs nor of lineage relationships between different cell types. Inner ear fate maps using lineage tracers are needed, but thus far only a few such studies have been performed (Fekete et al., 1998; Brigande et al., 2000a,b; Kil and Collazo, 2001; Lang and Fekete, 2001).

Single-cell lineage analysis using retroviral labeling in the avian hearing organ shortly before the last few cell divisions has revealed that HCs and supporting cells share common precursors (Fekete et al., 1998). This is what would be expected based on the knowledge that HCs regenerate in the adult bird's hearing organ and that their supporting cells can serve as precursors for new HCs. Although no direct hair cell-lineage study is available in the mammalian inner ear, it is generally believed that these cell types arise from a common origin in mammals as well.

Although much is already known about the molecular mechanisms regulating cellular specification and differentiation in the cochlea, especially in the organ of Corti (see next chapter), only fragmentary data about the mode and regulatory mechanisms of cochlear growth exist, both at the global and single-cell level.

Growth of the cochlea as of any other tissue depends on cellular proliferation, increase in cell diameter, cellular migration and death. The question arises as to how much each of these mechanisms contributes to cochlear growth? Active cellular migration has not been documented in the cochlear duct. In contrast to outgrowing nonsensory appendages (semicircular and endolymphatic ducts), cellular proliferation appears to primary account for the growth of the cochlear duct (Lang et al., 2000). Based on morphological signs, the auditory sensory epithelium originates from the ventromedial wall of the otocyst, but, as already pointed out, the identity and clonal relationship of the precursors giving rise to the organ of Corti with other sensory cell precursors of the early inner ear are unknown.

A region of high mitotic activity has been described in the ventromedial wall of the otocyst and this region has been suggested to be responsible for the elongation of the cochlear duct (Khan and Marovitz, 1982). The same region is thought to give rise to the sacculus. Routine histological analysis shows that there is considerable increase in the distance between the presumptive sacculus and the basal turn of the cochlear duct during development. However, from these data one cannot conclude that the cochlea grows exclusively at the junction between these two structures. Thus determining how the cochlear duct grows remains an important open question. Classic tritiated thymidine incorporation studies have shown that, in the mouse cochlea, cells of the organ of Corti (HCs and supporting cells) become postmitotic between embryonic days 12 and 15 (Ruben, 1967). Precursors giving rise to the organ of Corti are located in the ventral wall of the embryonic cochlear duct (Fig.

3A). This ventral wall is divided into two cellular ridges, termed as the greater (located medially) and lesser epithelial ridges, the GER and LER, respectively. Based on histological signs, it is thought that inner hair cells (IHCs) are derived from the GER and outer hair cells (OHCs) from the LER (Lim and Anniko, 1985). The suggestion that HCs are derived from the GER is supported by recent data showing that, in neonatal cochlear explant cultures, overexpression of *Math1* induces production of ectopic HCs in the GER (Zheng and Gao, 2000). However several questions remain, such as what is the location of the early, actively proliferating precursors of the organ of Corti, what are the molecular signals stimulating their proliferation, what are the modes of morphogenetic cell movements within the growing cochlear duct and what is the extent of cell mixing in this process? FGFs have been shown to promote precursor-cell proliferation in several tissues, such as stem cells in the developing telencephalon (Tropepe et al., 1999). Our data indicate that FGFR1 is required for the proliferation of precursors giving rise to the organ of Corti (paper VII). This is the first signaling mechanism shown to be required for the generation of this precursor-cell pool. The size of this progenitor population is subsequently determined by a cyclin-dependent kinase inhibitor, $p27^{Kip1}$, which regulates withdrawal of the precursors from the cell cycle. In addition to the cochlea, $p27^{Kip1}$ operates in the vestibular sensory epithelia, as shown by the $p27^{Kip1}$ null mutation that leads to hyperplasia of all inner ear sensory organs (Chen and Segil, 1999; Löwenheim et al., 1999). The GER of the embryonic cochlea regresses soon after birth. The inner sulcus of the adult cochlea (Fig. 3B and 3C) is a relic of the GER.

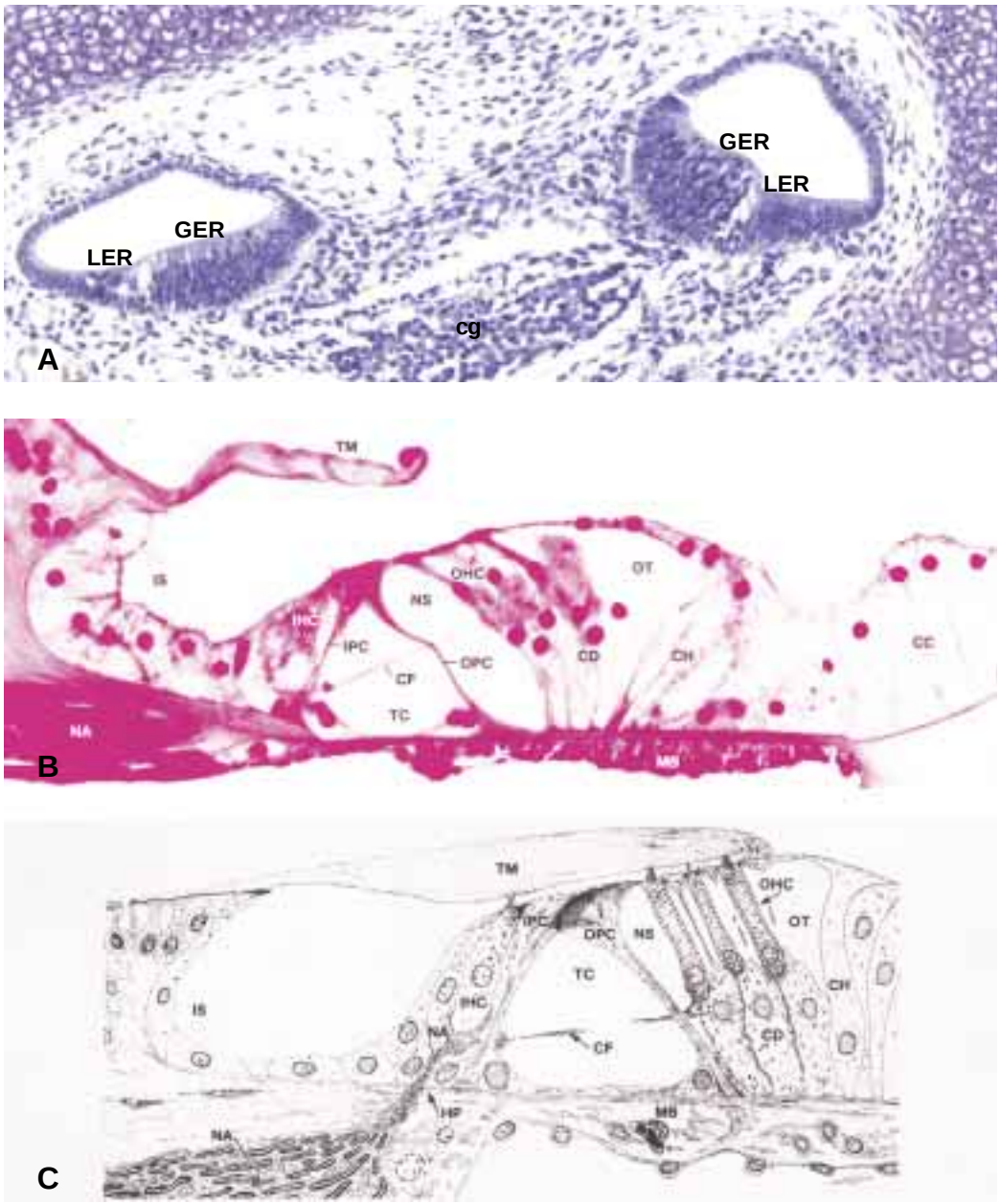

Figure 3. (**A**) Histological view of the embryonic day 16 mouse cochlea shown in a transverse section. The greater epithelial ridge is prominent. The lesser epithelial ridge of the middle coil (left) shows signs of hair cell differentiation whereas the apical coil (right) is more immature. Histological (**B**) and schematic (**C**) views of the

mature organ of Corti. Abbreviations: GER, greater epithelial ridge; LER, lesser epithelial ridge; cg; cochlear ganglion; TM, tectorial membrane; IS, inner sulcus; IHC, inner hair cell; OHC, outer hair cell; IPC, inner pillar cell; OPC, outer pillar cell; CF, tunnel crossing nerve fibers; TC, tunnel of Corti; NA, acoustic (cochlear) nerve; HP, habenula perforata; NS, Nuel's space; OT, outer tunnel of Corti; CD, Deiter's cells; CH, Hensen's cells; MB, basilar membrane; CC, Claudius cells. (B) and (C) from "The Inner Ear" (1983), van het Schip EP (ed), by permission from Duphar Nederland BV, Amsterdam.

4. Regulation of hair cell specification

The alternating pattern of HCs and supporting cells in the organ of Corti (Fig. 3B and 3C) and in the vestibular sensory epithelia has led to the hypothesis that local cell-cell communication rather than pre-programmed lineage-based mechanisms direct cell fate determination within the inner ear sensory epithelia. Recent studies support this hypothesis and suggest that the mechanosensory organs of flies, the sensory bristles and chordotonal organs (stretch receptors), are evolutionary homologous to the sensory patches and innervating neurons of vertebrates. There is conclusive evidence that all these structures use Notch signaling and members of the basic helix-loop-helix (bHLH) transcription factors in cell fate determination (reviewed by Eddison et al. 2000; Fritzsch et al., 2000).

The *Notch* pathway regulates cell-fate decisions in many organs both in invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999). In the

embryonic inner ear, *Notch* signaling regulates a process known as lateral inhibition in which neighboring precursor cells belonging to the same equivalence group adopt different fates (HCs or supporting cells). The receptor, Notch, interacts with the ligands, Delta and Serrate (also known as Jagged). The primary cell type (HC) expresses *Delta*, and this signaling is thought to increase Notch activity in the neighboring cells, causing the latter ones to adopt the alternative cell type (supporting cell). Gene mutations, both in the mouse and zebrafish, support the crucial role of Notch signaling in cellular specification in the inner ear. In the *mind bomb* zebrafish mutants in which Notch signaling is disrupted, the sensory patches consist of only HCs, which are produced in excess, while supporting cells are absent (Haddon et al., 1999). In the mouse, loss-of-function studies involving *Jagged2* and *Hes1*, which encodes a downstream effector of Notch activation (see below), show increased numbers of HCs, the expected result of disrupting lateral inhibitory mechanisms (Lanford et al., 1999; Zhang et al., 2000; Zheng et al., 2000).

The bHLH genes are evolutionary conserved, similar to the genes of the Notch pathway (Eddison et al. 2000; Fritzsch et al., 2000). Based on research on *Drosophila* sense organs, the proneural clusters (resembling the sensory patches of vertebrate ears), the regions of competence, are established first and the individual cell types are determined later. The fly's proneural clusters are defined by the expression of the proneural gene *Atonal*. Its expression is narrowed along with development to one or a few cell types. For example, during the formation of the ommatidia of the fly's compound eye, *Atonal* specifies the first-emerging

photoreceptor, termed as the R8 cell. This pioneering photoreceptor subsequently stimulates differentiation of the remaining photoreceptors and supporting cells by tyrosine kinase signaling (reviewed by Freeman, 1997). Thus, the formation of the fly's ommatidia is a good example of cellular specification and differentiation based on short-range intercellular communication.

In vertebrates, two *Atonal* homologs are crucial for inner ear development. *Neurogenin1* (*Ngn1)* is required for the generation of the cochlear and vestibular sensory neurons (see below). The other homolog is *Math1*. In the cochlea, *Math1* initially defines a broad region of the presumptive auditory sensory epithelium, but subsequently its expression becomes restricted to HCs. *Math1* is essential for the formation of both cochlear and vestibular HCs, as shown in the null mutants that lack all HCs (Bermingham et al., 1999*). Hes1*, a mammalian hairy and enhancer of split homolog, was initially characterized as a negative regulator of neurogenesis (Ishibashi et al., 1995). It has been suggested that Notch activation induces expression of *Hes*, which then antagonizes bHLH signaling (Ohtsuka et al., 1999). In an analogous manner, *Hes1* has been suggested to be a negative regulator of HC development in the inner ear (Zheng et al., 2000).

5. Regulation of hair cell differentiation

Brn-3c, encoding a POU-domain transcription factor, plays an essential role in the differentiation of both cochlear and vestibular HCs, as evidenced by the loss of all HCs in the null mutant mouse (Erkman et al., 1996; Xiang et al., 1997). Specifically, *Brn-3c* seems not to be involved in proliferation or fate determination of HC

precursors, because HCs are initially generated in normal numbers and they show initial signs of differentiation. However, HCs fail to form stereociliary bundles and they start to degenerate by apoptosis during late-embryogenesis (Xiang et al., 1998). Thus, *Brn-3c* is required for HC maturation. In *Brn-3c* null mice, HC loss leads to secondary degeneration of the innervating cochlear and vestibular sensory neurons. These observations support the notion that inner ear neurons are trophically dependent on their peripheral targets, the HCs.

Little direct evidences exists of the role of growth factors and their receptors during HC and supporting cell differentiation. It is known that FGFR3 signaling is essential for the formation of the cytoarchitecture of the organ of Corti. Specifically, *Fgfr3* null mice show a normal complement and normal morphology of cochlear HCs, but one type of supporting cells, the pillar cells, fail to differentiate. The lack of pillar cells prevents the formation of the tunnel of Corti, these defects leading to deafness (Colvin et al., 1996). These results clearly illustrate that, in addition to HCs, supporting cells are essential for hearing function. Our data show that, in the late-embryonic cochlea, *Fgf8* is expressed exclusively in IHCs, this expression starting at the onset of IHC differentiation. Additionally, *Fgf10* and *Fgf3* are expressed in the GER, in the region of IHCs (paper VII). We suggest that IHCs may induce differentiation of their later-emerging neighbours, the OHCs and supporting cells, through FGFR3 and FGFR1 signaling (Fig. 4; paper VII). Based on histological studies, it is well established that IHCs are the first cell types to start to develop within the organ of Corti. Together, sequential cellular differentiation within the auditory sensory epithelium seems to be regulated by FGF/FGR signaling, perhaps analogous to the

regulation of cytodifferentiation within *Drosophila* sense ogans by receptor tyrosine kinases (Freeman, 1997).

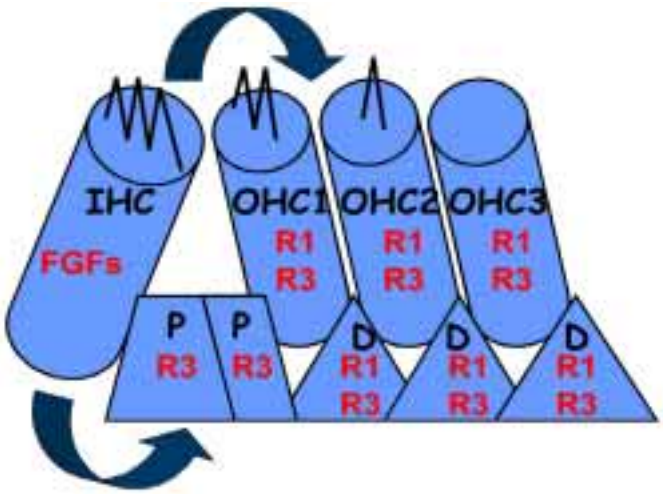

Figure 4. Schematic representation of FGF/FGFR signaling based intercellular communication within the differentiating organ of Corti. Inner hair cell-derived FGFs are suggested to act on FGFR-expressing outer hair cells and supporting cells. P, pillar cell; D, Deiter's cell, IHC, inner hair cell; OHC, outer hair cell; R1/R3, FGFR1/FGFR3.

Development of the apical mechanosensory region of HCs is an essential part of HC differentiation. This region comprises the stereocilia, which are deflected by acoustic stimulation, thereby initiating the transduction process, and the cuticular plate in which the stereociliary bundles are anchored. The mechanosensory region is packed with actin and associated cytoskeletal proteins. The maintenance and function of the elaborate cytoskeletal structure and the opening of the transduction channels are thought to be regulated by unconventional myosins, myosin VI, VIIA and XV. Mice carrying mutations in these genes show defects in stereociliary organization. Furthermore, defects in

myosin VIIA and *XV* are involved in human deafness (reviewed by Steel and Kros, 2001). In addition to their role in HC function, unconventional myosins are essential for organizing the stereociliary bundles during HC differentiation (Self et al., 1998; 1999).

6. Generation of inner ear sensory neurons

One of the earliest events of cell specialization in the inner ear is the delamination of precursors of the inner ear sensory neurons from the otic vesicle and their migration to form the cochleovestibular ganglion (D'Amico-Martel and Noden, 1983). Similarly as in the case of inner ear sensory organs, the origin of neuronal precursors in the otic epithelium is unknown and, thus, the possible lineage relationships are unresolved. It has been suggested that in parallel to the presumptive sensory organs, the singling out of precursors into a neural fate is regulated by Notch signaling and bHLH transcription factors (Adam et al., 1998). Similar to the inner ear sensory organs in which *Delta* is expressed in the HC precursors, it is expressed in the scattered cells in the otic epithelium that express neuronal markers and that delaminate to become neurons of the cochleovestibular ganglion. According to the principles of lateral inhibition, these *Delta*-expressing neuronal precursors are thought to inhibit their neighbors (expressing *Notch*) to become committed to a neural fate.

As in *Drosophila*, many bHLH transcription factors are crucial for vertebrate neurogenesis, including development of the neuronal compartment of the inner ear (Ma et al., 1998). Of the bHLH genes, *Ngns* regulate the earliest steps of

neurogenesis. In the peripheral nervous system (PNS), *Ngns* are essential for the initial selection of neuronal precursors within the sensory placodes and, thus, for neuroblast delamination from the placodal ectoderm. Similarly as in other cranial ganglia, all inner ear neurons fail to develop in *Ngn1* null mice (Ma et al., 1998, 2000). The delamination of neuronal progenitors from the neurogenic region within the otic vesicle is blocked in these mutants. *Neurogenins* are upstream activators of another bHLH gene, *NeuroD*, which promotes the withdrawal of precursors from the cell cycle and their differentiation into neurons (Lee et al., 1995). *NeuroD* null mice lack most of inner ear sensory neurons. This phenotype appears to be a combined effect of impaired delamination of neuroblast precursors and, since a small population neurons is initially formed, of the failure of early neurons to survive. Since expression of the neurotrophin (NT) receptors, *TrkB* and *TrkC*, is reduced in the early inner ear neurons of *NeuroD* null mutants, it has been suggested that unresponsiveness to NTs leads to neuronal apoptosis in these null mice (Liu et al., 2000; Kim et al., 2001).

In the PNS, neurons are initially produced in excess. More than 50% of peripheral sensory neurons, including those of the cochlear and vestibular ganglia, die during normal development. Neuronal survival depends on the availability of limiting amounts of neurotrophic factors secreted by the target cells that neurons innervate and for which they compete. Neurons that do not reach their peripheral targets die apoptotically. The inner ear serves as one of the best examples of the classic neurotrophic hypothesis formulated by Hamburger and

Levi-Montalcini (reviewed by Levi-Montalcini, 1987) stating that innervating neurons are dependent on their peripheral target fields. In the inner ear, two NT genes, *neurotrophin-3 (Nt-3)* and *brain-derived neurotrophic factor (Bdnf),* are expressed in the peripheral target fields, the sensory epithelia, and their tyrosine kinase receptors, *TrkC* and *TrkB*, are expressed in the cochlear and vestibular neurons (Fig. 5; Ernfors et al., 1992; papers I and II). *In vitro* experiments provided initial functional evidence that these NTs support survival of developing inner ear neurons and that they stimulate neurite outgrowth from the embryonic inner ear ganglia. Later, studies on transgenic mice have provided conclusive evidence for the pivotal role of the NT system in the inner ear (reviewed by Huang and Reichardt, 2001). When this trophic support is withdrawn by disrupting either *NT-3, BDNF, TrkB* or *TrkC* gene expression, large numbers of inner ear neurons are lost. In *Nt-3/Bdnf* double null mutant mice practically all inner ear neurons are lost (Ernfors et al., 1995).

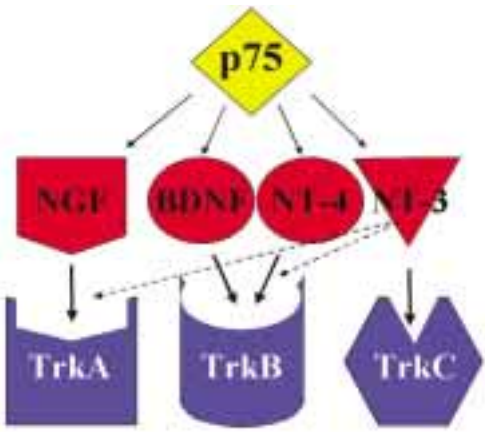

Figure 5. Schematic representation of the neurotrophin family ligand-receptor interactions. Primary interactions are indicated by thick arrows and secondary interactions by dashed arrows. All neurotrophins bind to low-affinity neurotrophin receptor, termed as p75.

II Hair cell regeneration in the adult inner ear

Following inner ear-specific traumas, exposure to noise or aminoglycoside antibiotics, neurons usually die secondarily following IHC loss. Therefore, in most cases HCs are the primary targets for therapeutic interventions. The adult mammalian ear lacks the plasticity that would allow natural HC regeneration. Once lost, HCs are lost forever leading to permanent hearing and balance deficits. This is in contrast to the ears of fish, amphibians and birds in which HCs are replaced following trauma. It has been shown in birds that new HCs become innervated by nerve fibers and that HC regeneration is accompanied by functional recovery. In response to noise and ototoxic drugs, supporting cells of the bird's hearing organ start to actively proliferate and transdifferentiate into new HCs. There is also evidence for direct transdifferentiation from supporting cells to HCs (reviewed by Cotanche, 1999). Over the past few years great efforts have been undertaken to identify mitogenic growth factors operating in the bird's inner ear, but the key factors are still unknown. Studies have been extended into the mammalian ear, testing exogenous growth factors and compounds acting on intracellular signaling pathways, but a "recipe" that could be used therapeutically to induce supporting cell proliferation in the adult mammalian ear *in vivo* is still to be found (Corwin and Oberholtzer, 1997). Stimulation of the regeneration process appears to be more difficult to accomplish in the mammalian cochlea as compared to their vestibular organs. Furthermore, although cellular proliferation is needed to compensate for the numbers of lost HCs and transdifferentiating supporting cells, additional strategies are likely to be needed to induce the transdifferentiation process. Thus, a combination of

molecules, which stimulate HC proliferation and differentiation might form an effective therapeutic intervention. As said, these compounds are still to be found as are also the optimal delivery method(s) into the inner ear.

III Trauma-induced cell death and its regulation

In addition to attempting to regenerate HCs, another therapeutic approach might be to prevent or attenuate trauma-induced HC death. The ability to prevent HC damage has in part been unattainable because the molecular mechanisms of noise- and ototoxic drug-induced HC trauma are not well understood. Recent data have suggested that, in response to traumas, a considerable population of HCs die through apoptosis (Forge, 1985; Li et al., 1995; Liu et al., 1998; Nakagawa et al., 1998; Vago et al., 1998; Hu et al., 2000; papers IV, V). Given the apparent central role of apoptosis in many diseases and traumas, not only those of the inner ear, intense interest has been focused in understanding the molecular mechanisms behind this process. This knowledge opens avenues for developing diagnostic, prognostic and therapeutic tools. One potential apoptosis-based therapy is the use of small pharmaceutical compounds designed to act on upstream modulators of apoptosis. However, rescuing a cell from death does not necessarily equate to preserving its function.

1. Modes of cell death

Apoptosis is one of the major forms of cell death. In the developing nervous system, it regulates morphogenetic processes, such as neural tube closure, formation of the correct size of neuronal populations (the neurotrophic hypothesis) and the

establishment synaptic connections. In adults, apoptosis contributes to homeostasis and to many pathologies, including environmental stress-induced trauma and neurodegenerative disorders. Cell morphological manifestations of apoptosis include cellular shrinkage, chromatin condensation and DNA fragmentation and finally cellular fragmentation into small apoptotic bodies that are phagocytosed by adjacent cells (Kerr et al. 1972; Wyllie et al. 1980). Apoptosis is a gene-directed self-destruction process, a regulated mode of cell death that results from the endogenous *de novo* protein synthesis or posttranslational activation of a set of proteins that are involved in intracellular signaling cascades (Raff 1992; Weil et al. 1996). Necrosis in another type of cell death, based on morphological and biochemical criteria. It is thought to result from more passive mechanisms triggered by extrinsic insults such as trauma, toxins, microbes. The apoptosis versus necrosis classification has been useful in categorizing cell death in numerous settings, but the relationship between these modes of death is not always clear. For example, following excitotoxic or anoxic-ischemic injury some subsets of neurons undergo apoptosis, whereas others undergo necrosis. Some reports suggest that biochemical features of apoptosis and morphological evidence of necrosis can be observed even in the same individual neurons of the adult brain (Portera-Caillau et al. 1997a,b). Thus, in pathological situations, evidence suggests that an apoptosis-necrosis morphological cell death continuum exists. However, biochemical features clearly separate apoptosis from necrosis. Although necrotic death also has been shown to be accompanied by a loss of mitochondrial membrane potential (Matsumura et al., 2000), the release of cytochrome c from mitochondria, regulated by the

Bcl-2 family of proteins, and the activation of the caspase cascades are thought to be exclusively biochemical features of the apoptotic machinery (reviewed by Gross et al., 1999).

2. c-Jun N-terminal kinase signaling

Cells respond to changes in physical and chemical properties of their environment. Intracellular mitogen-activated protein kinase (MAPK) pathways play an important role in the decision making processes, which make cells proliferate, migrate, differentiate or die. Many cell types respond to environmental stress by activation of one of the MAPK pathways, the c-Jun N-terminal kinase (JNK) cascade, which is often called the stress-kinase cascade. JNK cascade consists of series of kinases, which become sequentially activated (phosphorylated). Activation of JNKs, the central modules of this cascade, lead to the phosphorylation of their target, the nuclear transcription factor c- Jun (reviewed by Davis, 2000; Fig. 6). JNK pathway has been shown to couple external stimuli to apoptosis. In particular, the proapoptotic role of JNK signaling has been shown in neurons exposed to various stresses, including growth factor withdrawal, excitotoxicity and reactive oxygen species (ROS) (Xia et al., 1995; Yang et al., 1997; Lo et al., 1996). Successful tools in elucidating the biochemical and functional roles of the JNK pathway have been the inhibitors that act on components in the cascade. These include dominant negative mutant constructs that can block growth factor withdrawal-induced apoptosis of cultured neurons (Estus et al., 1994; Ham et al., 1995; Xia et al., 1995; Xu et al., 2001). Another approach has been the use of a specific pharmacological inhibitor of the JNK cascade, CEP-1347. This indolocarbatzole has been shown to protect various

cell types from stress-induced death both *in vitro* and *in vivo* (Maroney et al., 1998; Glicksman et al., 1998; Saporito et al., 1998, 1999; papers IV and V).

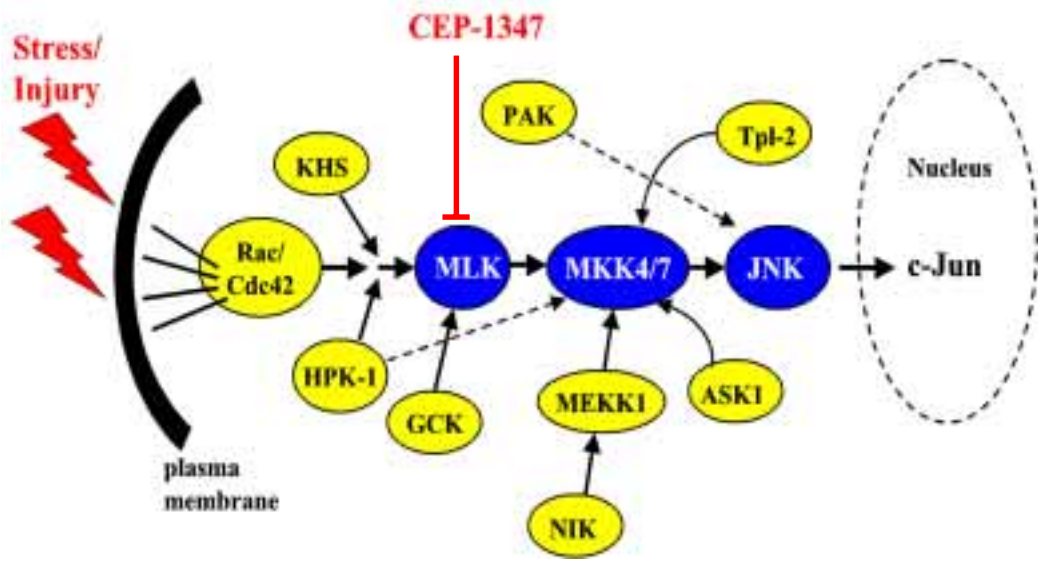

Figure 6. Stress-activated JNK pathway. The central MAPKKK (MLK), MAPKK (MKK4/7), MAPK (JNK) molecules are coloured blue. c-Jun, the target of the kinases, is a nuclear transcription factor. Other kinases modulating the central components are coloured yellow as are also the Rac/Cdc42 GTPases locating near the cell membrane and mediating JNK activation.

One of the first direct demonstrations of a mediator role of JNK signaling in apoptosis was the finding that targeted inactivation of the *Jnk3* gene, which is primarily expressed in the brain, protects hippocampal neurons against excitotoxic death (Yang et al., 1997). This finding is supported by the

demonstration that genetic inhibition of activation of c-Jun, the nuclear target of JNK, confers hippocampal neuron protection against kainate-induced apoptosis (Behrens et al., 1999). Also ultraviolet (UV)-induced apoptosis of fibroblasts is mediated by the JNK cascade, as shown in *Jnk1*/*Jnk2* double knock out mice (Tournier et al., 2000). Fibroblasts derived from these mutant mice are protected against UV-stimulated apoptosis. Importantly, by using these mutant cells it has been shown that JNK signaling is required for the activation of the effector apparatus of apoptosis, including cytochrome c release from mitochondria and activation of caspases (Tournier et al., 2000). JNK activation does not apparently directly induce cytochrome c release, but members of the Bcl-2 family of apoptotic regulatory proteins may mediate this effect (reviewed by Davis, 2000). Consistent with the suggestion that JNK signaling acts upstream of the mitochondrial events leading to apoptosis, overexpression of mixed-lineage kinases (MLKs), that are located above JNKs in the JNK signaling cascade, can induce neuronal death. Whereas their dominant-negative forms prevent neuronal death caused by growth-factor deprivation (Xu et al., 2001).

In addition to stress-induced apoptosis, JNK signaling has been shown to regulate many aspects of development. Although the role of JNKs during embryogenesis has been mainly elucidated in *Drosophila*, there exist some data providing direct evidence on their role during mammaliam morphogenesis. *Jnk1/Jnk2* double mutation causes early embryonic lethality due to severily reduced apoptosis in the early hindbrain, which inhibits neural tube closure (Kuan et al., 1999). It is important to point out

that although JNK signaling has been shown to mainly exert proapoptotic effects, it can also have antiapoptotic effects. For example, in the *Jnk1/Jnk2* double mutant mice, described above, apoptosis is reduced in the early hindbrain, but increased in the forebrain (Kuan et al., 1999). Thus, the consequence of JNK activation on apoptosis may depend on the cell context and on the combination of simultaneously activated signaling pathways within a cell (Ip and Davis, 1998). This seems to be especially important during environmental stress.

AIMS OF THE STUDY

The aim of the present study was to investigate the molecular regulation of the development and death of inner ear hair cells and neurons.

The specific aims were:

- to characterize the neurotrophin system of the rat inner ear by gene expression studies and *in vitro* assays.
- to characterize the neurotrophin system in the chick inner ear with the specific aim of studying the possible similarities between the innervation process during embryogenesis and reinnervation following trauma-induced regeneration during adulthood.
-to characterize the mode(s) of HC death following traumas and to reveal the functional significance of the JNK cascade.
- to study the functional significance of FGF/FGFR2(IIIb) and FGF/FGFR1 signaling in the developing inner ear by transgenic technology.

MATERIALS AND METHODS

Animals

The use of the experimental animals and the experimental procedures were approved by the Committee of Animal Experiments at the University of Helsinki. Embryonic Sprague-Dawley (papers I and II) and Wistar (paper IV) rats, embryonic and postnatal White Leghorn chicks (paper III), adult Dunkin-Hartley guinea pigs (papers IV and V) and early postnatal BALB/c mice (paper V) were used in experiments.

In addition, several mutant mouse lines were studied (papers VI and VII). Generation of the *Fgfr2(IIIb)* knock out mice has been described by DeMoerlooze et al. (2000). The $Fgfr1^{n7/n7}$ and $Fgfr1^{n15YF/n15YF}$ hypomorphic mice lines were produced by Partanen et al. (1999). The generation of the mutant mice carrying the $Fgfr1^{flox}$ allele is described in paper VII and in more detail by Trokovic et al. (submitted manuscript). The *Foxg1-Cre* mice were generated by Hébert and McConnell (2001). Generation of the $Fgfr1^{\Delta flox/flox}$*; Foxg1-Cre/+* conditional mutants is described in paper VII. The *D4/XEgfp* mice carrying a X-chromosomal *Egfp* transgene have been described by Hadjantonakis et al. (1998). Genotyping was done from tail samples by PCR or, in the case of *D4/XEgfp mice*, by EGFP immunohistochemistry.

Lesions

Three-week-old chicks were exposed to noise with a center frequency of 1.5 kHz at 120 dB SPL for 20 hrs as described in paper III. Adult guinea pigs

were exposed to octave band noise with a center frequency of 4 kHz, 120 dB SPL for 6 hrs as described in paper IV. In paper V, adult guinea pigs were injected with gentamicin (120 mg/kg body weight), once daily for 14 days.

Hearing tests

Auditory brainstem responses (ABRs) were measured from guinea pigs before (baseline values) and after traumas. ABRs were measured under light anesthesia (xylazine, 10 mg/kg; ketamine, 40 mg/kg). The method is described in papers IV and V and by Ylikoski et al. (1998).

Cytocochleograms from adult cochleas

Auditory HCs were counted from cochlear surface specimens. Adult guinea pig inner ears were fixed with 2.5% glutaraldehyde (GA), postfixed with 1% osmium tetroxide and embedded in Epon. The preparation of cytocochleograms is originally described by Ylikoski et al. (1974a). The method of HC counting under Nomarski optics is described in papers IV and V.

Wholemount cochlear surface specimens from developing cochleas

At late-embryogenesis and early postnatal life, HCs numbers were analysed from PFA-fixed and rhodamine phalloidin-stained wholemount cochlear surface specimens. Phalloidin detects F-actin, which is abundant in the apical mechanosensory region of HCs and in the apical processes of the adjacent supporting cells. The dissection technique is described in papers IV and VII.

Preparation of paraffin sections and semithin sections

For *in situ* hybridisation, immunohistochemistry, cell death and cell proliferation assays, embryonic and adult inner ears were fixed with PFA. Following fixation, adult inner ears were decalcified with 0.5 M ethylenediaminetetraacetic acid. Specimens were embedded in paraffin and cut to 5-µm-thick sections. For semithin sections (papers III, IV, V and VII), specimens were fixed with GA and osmium tetroxide, embedded in Epon and cut to one-µm-thick plastic sections.

The protocol of **radioactive *in situ* hybridisation** using paraffin sections and ^{35}S-labeled cRNA probes is originally described by Wilkinson and Green (1990). We used this protocol with minor modifications described in papers I, II and III. Probes used are listed in Table 1. The protocol of **nonradioactive *in situ* hybridisation** on paraffin sections using digoxigenin-labeled cRNA probes is described in paper III.

Immunohistochemistry was performed on paraffin sections. To detect primary antibodies, the avidin-biotin-peroxidase method (Vectastain Elite ABC kit by Vector Laboratories) and diaminobenzidine (DAB) were used. Sections were counterstained with methyl green. In the case of phospho-specific JNK and c-Jun antibodies, immunostainings were amplified using tyramide signal amplification (TSA-Indirect kit by New England Nuclear). It was performed according to manufacturer's recommendations with the modifications described in paper IV.

Cellular proliferation was detected by 5'-bromo-2´-deoxyuridine (BrdU) incorporation as described in papers II and VII. Briefly, pregnant rats (paper II)

and mice (paper VII) received a 2-hr-long BrdU pulse. BrdU was also injected to noise-exposed chicks according to the regimen described in paper III. Dividing cells were detected from paraffin sections by BrdU antibodies, followed by the indirect immunoperoxidase method using DAB as the substrate.

Apoptotic cell death was detected by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-method. A commercial TUNEL-kit using fluorescein-based detection was used on paraffin sections according to manufacturer's instructions (papers IV, V, VI, VII)

In vitro assays

Neuron-enriched dissociated cultures from embryonic cochleovestibular ganglia (papers II and III) and cochlear ganglia (paper IV) were prepared according to the method by Davies and Lindsay (1985) with the minor modifications described in paper II. The response to NTs and CEP-1347 was assessed 48 hrs after plating by counting the numbers of phase-bright neurons with distinct neurites.

Embryonic cochlear and vestibular **ganglion explants** were cultured in collagen matrix according to the method by Ebendal (1989). After a 48-hr-long culture period with or without NTs, the explants were fixed with PFA and immunostained as wholemounts with neurofilament antibodies. The magnitude of neurite outgrowth was scored under fluorescence (papers II and III) or phase-contrast (paper I) optics.

Cochlear explant cultures

Cochleas of postnatal day 2 rats and mice were dissected and maintained for 48 to 72 hrs *in vitro* as described in paper IV. We adopted the organotypic culture method developed by Saxén and collegues who used it for embryonic kidneys (Saxén, 1987). Briefly, part of the otic capsule is removed and the cochlear duct is placed on a piece of filter paper (Nuclepore, pore size 0.1 μm, Corning), which in turn is placed on a Trowell-type metal screen (Fig. 7). The screen is placed in a culture dish and culture medium is added up to the level of the screen. In our experience, the developing organ of Corti favours these conditions as compared to the many other cochlear explant culture techniques used.

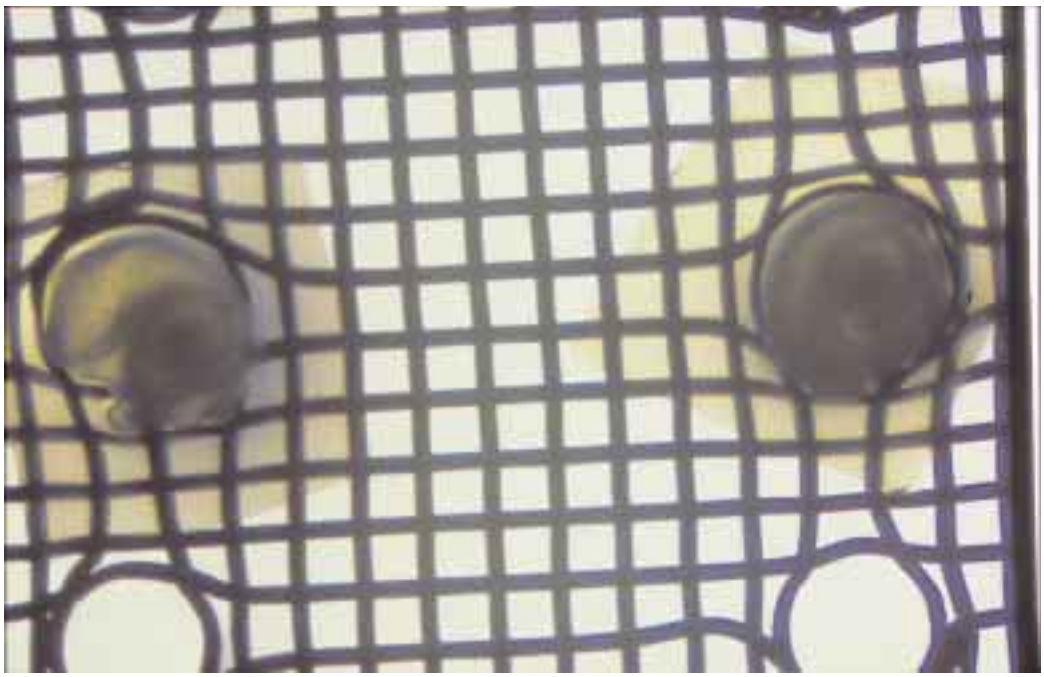

Figure 7. *C*ochlear explants in a Trowell-type organotypic culture.

Table 1. Probes used for *in situ* hybridisation.

Probe	Species	Reference
Bdnf	rat	paper I
Nt-3	rat	paper I
Nt-4/5	rat	paper I
Ngf	rat	paper I
p75	rat	paper II
TrkA	rat	paper II
TrkB pan	rat	paper II
TrkB catalytic	rat	paper II
TrkB T1 truncated	rat	paper II
TrkC pan	rat	paper II
Neurofilament 68	rat	paper II
Bdnf	chicken	Hallböök et al., 1993
Nt-3	chicken	Hallböök et al., 1993
Ngf	chicken	Hallböök et al., 1993
TrkB pan	chicken	Dechant et al, 1993
TrkB catalytic	chicken	Dechant et al, 1993
Fgf10	mouse	Bellusci et al., 1997
Fgf3	mouse	Wilkinson et al., 1989
Fgfr2 (pan)	mouse	Orr-Urtreger et al., 1991
Fgfr2(IIIb)	mouse	Orr-Urtreger et al., 1993
Fgfr2(IIIc)	mouse	Kettunen et al., 1998
Pax2	mouse	Dressler et al., 1990
Fgfr1 (pan)	mouse	Orr-Urtreger et al., 1991
Fgfr1 “floxed”	mouse	Trokovic et al., submitted
Fgfr1(IIIb)	mouse	Kettunen et al., 1998
Fgfr1(IIIc)	mouse	Kettunen et al., 1998
Fgfr3	mouse	Peters et al., 1993
Delta1	mouse	Morrison et al., 1999
Serrate1	mouse	Morrison et al., 1999
Math1	mouse	Helms and Johnson, 1998
Fgf8	mouse	Heikinheimo et al., 1994

RESULTS AND DISCUSSION

1. Target-derived neurotrophins promote survival and differentiation of sensory neurons of the rat inner ear (papers I and II)

Nerve growth factor (NGF) was discovered more than 50 years ago as a molecule that promotes neuronal survival and differentiation in the PNS (reviewed by Levi-Montalcini, 1987). The next neurotrophin identified was BDNF, which was shown act on partially different subsets of neurons than NGF (Barde et al., 1982). Between 1989 and 1991, *Bdnf*, *Nt-3* and *Nt-4* cDNAs were cloned and were shown to possess a high degree of structural homology to *Ngf*. At the same time period, the cloning of three members of the Trk receptor tyrosine kinase family, *TrkA*, *TrkB* and *TrkC*, was published. These receptors bind NTs with high-affinity and initiate the intracellular signal transduction process (reviewed by Huang and Reichardt, 2001; Fig. 5). The neuronal survival- and differentiation-promoting activities of NTs were first demonstrated in two types of classic *in vitro* assays, the survival assay using dissociated neurons from the embryonic PNS (Davies and Lindsay, 1985) and the neurite outgrowth assay using ganglionic explants cultured in three-dimensional collagen matrix (Ebendal, 1989) (reviewed by Huang and Reichardt, 2001).

All *Trk* genes are alternatively spliced. For example, three TrkB isoforms have been identified, which differ in their cytoplasmic domains. The full-length (catalytic) isoform contains a cytoplasmic tyrosine kinase domain whereas it is replaced by a short sequence in the truncated isoforms. Most part of this truncated intracellular domain lacks obvious homology to any known protein

motifs (reviewed by Barbacid, 1994). Full-length *TrkB* is almost exclusively detected in neurons whereas truncated receptors can be found both in neurons and non-neuronal cells. Similar to full-length TrkB, truncated TrkB receptors can bind and internalize BDNF. They have been suggested to act as negative regulators of full-length TrkB signaling, to regulate the availability of BDNF to responsive systems and to modulate neuritic growth (Klein et al., 1990; Beck et al., 1993; Biffo et al., 1995; Eide et al., 1996; Yacoubian and Lo, 2000). However, despite the many suggested functions, direct evidence of the roles of truncated TrkB receptors *in vivo* remains to be shown.

In paper I, we suggested that, in contrast to earlier reports, *Ngf* is not expressed in the sensory structures of the inner ear and that NGF does not elicit neurite outgrowth from the inner ear ganglia *in vitro*. Instead, we showed that *Nt-3 (Fig. 8)* and *Bdnf* are expressed in the developing inner ear sensory epithelia and that the encoded proteins, but not NGF, induce neurite outgrowth from the cochleovestibular ganglion explants. These were actually expected results, since NGF had earlier been shown to act on neural crest-derived, but not placodal-derived sensory neurons (Davies and Lindsay, 1985). We demonstrated the partially overlapping expression patterns of *Nt-3* and *Bdnf* in the developing organ of Corti, *Bdnf* signal being restricted to IHCs and OHCs, whereas *Nt-3* mRNA was found in the surrounding supporting cells too. In the vestibular organs, *Nt-3* is expressed in the saccular and utricular maculae, both in HCs and supporting cells. *Bdnf* is expressed in the HCs of the sacculus and utriculus, and, in contrast to *Nt-3*, also in the HCs of the ampullary cristae of the three

semicircular ducts. We also showed that these two *NT* genes start to be expressed in the presumptive sensory patches of otic vesicle before nerve fibers innervate these regions. These initial observations supported the classic hypothesis of dependance of innervating neurons for survival and differentiation on their peripheral target field. Results similar to our data were concomitantly reported by another group (Ernfors et al., 1992).

In paper II, we showed that *TrkB* and *TrkC (Fig. 9),* coding for the signaling receptors for BDNF and NT-3, are expressed in the cochlear and vestibular neurons. These mRNAs are expressed in the cochleovestibular ganglion from the beginning of its formation. In neuron-enriched dissociated cultures, NT-3 and BDNF, but not NGF efficiently supported the survival of cochleovestibular neurons. When NT-3 and BDNF proteins were added in combination to the culture medium, an overlapping rather than additive effect was found. These results were in agreement with the *in situ* hybridisation data demonstrating coexpression of *TrkB* and *TrkC* in these neurons. This receptor coexpression has been later verified by immunohistochemistry (Farinas et al., 2001).

Our expression studies and *in vitro* assays suggested that NT-3 and BDNF and their cognate receptors are required for the establishment of the afferent innervation of the inner ear. The development of gene targeting technology provided a crucial tool to test the validity of these suggestions. Table 2 shows the percentages of lost inner ear neurons reported by different research groups in the different *neurotrophin* and *Trk* knock out mice. In many of these mutants, the inner ear was found to be

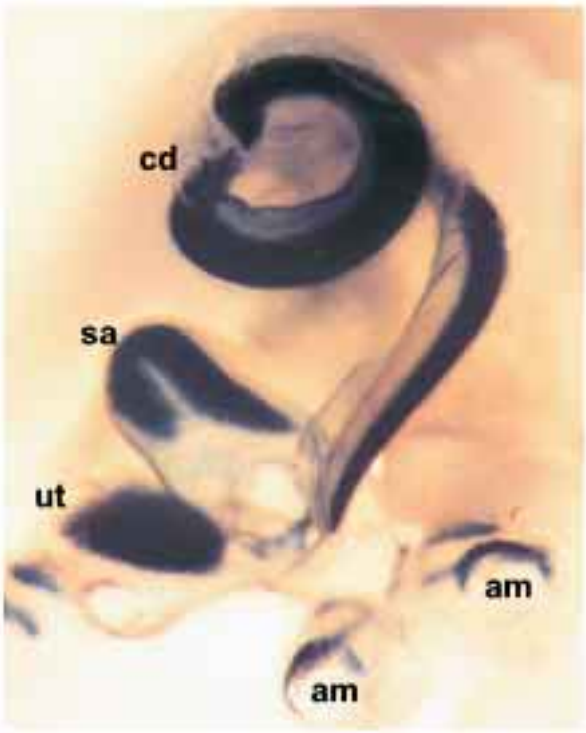

Figure 8. *Nt-3* expression in the inner ear sensory epithelia of a knock out mice at birth as revealed by lacZ staining. We are greatful to Dr. L.F. Reichardt for donating the *Nt-3* mutant mouse line to us. Abbreviations: cd, cochlear duct; sa, saccular macula; ut, utricular macula; am, ampullary crista.

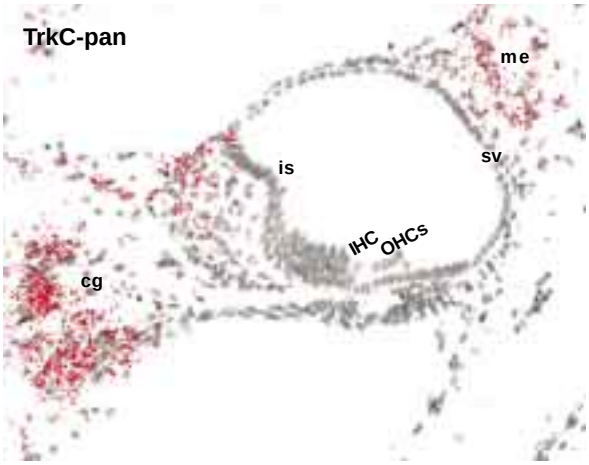

Figure 9. *TrkC* expression in the rat cochlea at birth as revealed by a pan-*trkC* probe. By using isoform-specific probes, we have found that the full-length (catalytic) isoform is expressed exclusively in the cochlear neurons, whereas the truncated isoform is expressed in non-neuronal locations, particularly in the mesenchyme (me). Note that the organ of Corti, including the inner hair cells (IHC) and outer hair cells (OHCs) is devoid of *TrkC* expression. Abbreviations: cg, cochlear ganglion; is, inner sulcus; sv, stria vascularis; me, mesenchyme.

one of the most severely affected organs. The results show that NT-3 and BDNF exert partially complementary survival-promoting roles, NT-3 predominating in the hearing organ and BDNF in the vestibular organs. Since *Nt-3* inactivation causes a more severe phenotype than *TrkC* deletion, NT-3 might also activate its "non-preferred" TrkB receptor. Direct evidence for this suggestion has been recently shown in other sensory ganglia (Farinas et al., 1998; Huang et al., 1999). In *Nt-3, Bdnf, TrkB* and *TrkC* null mice, inner ear neurons are initially formed, but they die by apoptosis during late-embryogenesis. Taken together, data from gene disruption studies are in agreement with the original expression data and *in vitro* assays, all these data pointing to the pivotal role of NT-3 and BDNF in the generation of the neuronal compartment of the inner ear.

Table 2. Neuronal losses in the inner ear of *neurotrophin* and *Trk*-deficient mice.

Ganglion	***Nt-3-/-***	***Bdnf-/-***	***Nt-3-/- Bdnf-/-***	***Ngf-/-***	***Nt-4/5-/***	***TrkC-/-***	***TrkB-/-***	***TrkC-/- TrkB-/-***	***TrkA-/-***
cochlear	85[a,c]	7[c]	100[c]	ND	NO[e,f]	50-70[g,h]	15[g,h]	61[h]	NO[j]
vestibular	20-35[a,c]	80[b,c,d]	100[c]	ND	NO[e,f]	15[g,h,i]	56[g,h]	58[h]	NO[j]

[a]Farinas et al., 1994; [b]Ernfors et al., 1994; [c]Ernfors et al. 1995; [d]Bianchi et al., 1996; [e]Conover et al., 1995; [f]Liu et al., 1995; [g]Schimmmang et al., 1995; [h]Minichello et al., 1995; [i]Tessarollo et al., 1997; [j]Huang and Reichardt, 2001. ND, not done; NO, not observed.

An interesting approach has been recently used to further dissect NT functions. The *Nt-3* gene has been replaced by *Bdnf* (Coppola et al., 2001; Farinas et al., 2001). Thus, in this strategy signaling by TrkC is eliminated, while TrkB signaling is preserved (by the ectopic *Bdnf*). It results in almost complete rescue of the cochlear neuron

loss caused by *Nt-3* disruption. This is what would be expected based on the data showing that *TrkB* and *TrkC* receptors are colocalized in cochlear neurons (papers I and II; Farinas et al., 2001).

In papers I and II, we show that the thickened sensory patches of the otic vesicle, which comprise the common precursors for HCs and supporting cells, are prominently labeled with the *Nt-3* probe, perhaps reflecting later expression of this gene in both differentiating HCs and supporting cells. *Bdnf* expression in the sensory patches of the vesicle appeared less prominent. We do not know whether these differences in expression levels reflect an early fate segregation of the common precursors as HCs and supporting cells. The stage at which these cellular fates become determined is currently unknown. Expression of *neurotrophin*s in the otic epithelium preceded compaction of the cochleovestibular ganglion and also *TrkB* and *TrkC* expression in the neurons, indicating that the induction of *Nt-3* and *Bdnf* expression is independent of the neuronal compartment. In general, only little is known about the genetic pathways regulating *NT* induction during embryogenesis. It has been suggested recently that, in the developing limb bud, ectoderm-derived WNT factors can induce *Nt-3* expression in the underlying mesenchyme (Patapoutian et al., 1999).

Based on spatiotemporal expression patterns, we suggested that NTs produced by the sensory patches of the otocyst might act as attractants for the primary neurites from the cochleovestibular ganglion (papers I and II). The *TrkB* and *TrkC* receptors start to be expressed in the ganglion before neurites penetrate

into the otic epithelium. However, we have not provided direct *in vitro* evidence for this putative chemoattractant role. Since NTs are highly basic molecules, *in vivo*, they are likely to be bound to the ECM and cell surfaces near the site of diffusion. Thus, they might act as short- range rather than long-range guiding cues for the growing neurites. Ingrowth of nerve fibers into the otic epithelium is followed by the formation of synaptic contacts with the early-differentiating HCs. *Nt-3* is expressed both in HCs and supporting cells, but *Bdnf* is found exclusively in HCs. Therefore, BDNF might have a more important role in guidance of the neuritic growth cones towards their synaptic targets as compared to that of NT-3.

Our other paper (Ylikoski et al., 1993) shows that expression of *Bdnf* , *Nt-3, TrkB* and *TrkC* persist in the HCs of the early postnatal cochlea. By the end of the second postnatal week, *Bdnf* expression is turned off in the cochlea whereas *Nt-3* expression is shifted to the IHCs only. This time period when qualitative and quantitative changes occur in *NT* expression in the cochlea coincides with the remarkable remodeling of the innervation at the nerve terminal level. Importantly, all these changes shortly precede the onset of hearing function. In the remodeling process, afferent innervation is shifted mainly to IHCs (only 5 to 10% of cochlear neurons thereafter innervate OHCs) whereas the majority of efferent fibers terminate on OHCs. Expression data suggests that BDNF and NT-3 might regulate this process, perhaps together with GDNF (Ylikoski et al., 1998).

As stated above, in the adult cochlea, *Nt-3* is expressed exclusively in IHCs (Ylikoski et al., 1993). Combined with the fact that IHCs receive the majority of afferent innervation in adults, these data suggest that target-derived, retrogradely transported NT-3 might act on the *TrkC* and *TrkB*-expressing cochlear sensory neurons also during adulthood. In the adult vestibular system, *Nt-3* and *Bdnf* and their cognate receptors have similar expression patterns as during development (Ylikoski et al., 1993). A neuroprotective role for the two NTs during adulthood has been suggested. Data from other adult neuronal systems indicate that, in most cases, mature neurons lose absolute dependence on target-derived growth factors for acute survival and that NT function appears to shift from the regulation of neuronal survival to the regulation of phenotype and function. However, following NT withdrawal, most neuronal populations of the PNS gradually show atrophic changes and die within a few weeks or months. Also adult cochlear neurons show progressive retrograde degeneration following IHC loss (Spoendlin, 1971; Ylikoski et al., 1974b; Webster and Webster, 1981). For example in guinea pigs, destruction of IHCs by intense noise exposure or aminoglycosides results in the loss of the majority of auditory neurons by four weeks postexposure (Ylikoski et al., 1974b, 1998). The obvious cause for this death is the loss of trophic support from the targets cells, the HCs, as evidenced by *in vivo* studies in which local infusion of recombinant NT-3, BDNF or GDNF proteins into the noise- or ototoxic drug-exposed cochlea can prevent neuronal death (Ernfors et al., 1996; Staecker et al., 1996; Miller et al., 1997; Ylikoski et al., 1998). These data indicate that neurotrophic factors may have therapeutic potential especially in cases where

neuronal protection is the primary aim, such as in conjunction with cochlear implant insertions.

2. Neurotrophin signaling in the developing and regenerating avian hearing organ (paper III)

We were motivated to study the role of NT signaling in the chick's hearing organ, because, in contrast to the mammalian cochlea, lost HCs in birds are replaced by new ones and this regeneration is followed by functional recovery. Available data showed that new HC formation in adult chicks is followed by the establishment of synaptic connections (Umemoto et al., 1995; Wang and Raphael, 1996), but nothing was known about the molecular mechanisms directing the reinnervation process. This process seemed to recapitulate some features of the embryonic innervation process. We hypothesized that similarities could also been seen at the molecular level.

We found distinct qualitative differences between the NT system in the avian and rodent cochlea. In the cochlea of the latter species, *Nt-3* is the predominant *NT*, whereas it is hardly expressed in the avian cochlea. Only very weak *Nt-3* expression could be found in the chick´s auditory organ and it was restricted to the earliest stages of cochlear formation. In contrast, we found distinct *Bdnf* expression in the differentiating and adult cochlear HCs. *TrkB* was detected in the innervating neurons throughout life. We also documented the survival- and neurite outgrowth-promoting effects of recombinant BDNF on embryonic chick's cochleovestibular neurons *in vitro*. Combining the expression of *NTs* in the mammalian (papers I and II; Ernfors

et al., 1992; Ylikoski et al., 1993), chick (paper III; Hallböök et al., 1993) and *Xenopus* (Don et al., 1997) inner ear, it appears that the evolutionary older vestibular system depends on BDNF signaling whereas the acquisition of a specialized, spiraling hearing organ, as seen in mammals, is accompanied by an implementation of another neurotrophin, NT-3.

We used noise exposure to destroy part of the chick´s auditory HCs and to induce the formation of new ones. As analysed one week after acoustic overstimulation, induction of regeneration was documented by supporting cell proliferation and histologically by showing the presence of immature HCs. New HCs are known to arise through proliferation and transdifferentiation of the underlying supporting cells. Supporting cells did not express *Bdnf*, but this signal was upregulated in the new HCs. *Bdnf* induction was seen already in the early-differentiating HCs. We found prominent expression of the truncated *TrkB* receptor in supporting cells, but this expression was turned off along with the transdifferentiation process. Based on the suggestions that truncated TrkB isoforms can bind and internalize BDNF (Klein et al., 1990; Biffo et al., 1995) and regulate neuritic growth (Beck et al., 1993; Yacoubian and Lo, 2000), they might facilitate the regrowth of nerve endings into contact with the differentiating HCs. Thus, this might be a mechanism accounting for the rapid innervation of regenerated HCs, a phenomenon documented in other studies (Umemoto et al., 1995; Wang and Raphael, 1996). This knowledge obtained from the chick cochlea gives hope that reinnervation might be possible also in the mammalian cochlea if the tools to stimulate mammalian HC regeneration are finally realized.

3. The c-Jun N-terminal kinase pathway mediates stress-induced hair-cell death (papers IV and V)

Noise and aminoglycoside antibiotics are fatal to HCs. Apoptotic HC death has been documented following both traumatizing agents, based on morphological criteria and the TUNEL-method that labels fragmented DNA of dying cells (Forge, 1985; Li et al., 1995; Liu et al., 1998; Nakagawa et al., 1998; Vago et al., 1998; Hu et al., 2000; papers IV, V). In paper IV, we show by TUNEL-staining and by histological analysis of semithin sections that a population of cochlear HCs of adult guinea pigs die by apoptosis following the noise exposure paradigm used (120 dB SPL, 4 kHz, 6 hrs). We also show the presence of apoptotic HCs in neomycin-exposed cochlear explants. In paper V, we show vestibular HC apoptosis following gentamycin-exposure *in vivo*. However, we would not like to make a clearcut distinction between the morphologies associated with apoptotic and necrotic cell death. Especially in pathological situations the distinction between the two modes of death is not always clear. This may well be the case in intense noise exposures or when applying very high doses of ototoxic drugs.

Increasing evidence from a variety of cells and tissues points to a role for JNK signaling in mediating stress-induced apoptosis. Activation of the JNK cascade involves sequential phosphorylation of kinases and finally phosphorylation of the c-Jun transcription factor (reviewed by Davis, 2000). By using phospho-specific JNK and c-Jun antibodies in immunohistochemistry, we documented the activation of JNK cascade in HCs of neomycin-exposed cochlear explants

(paper IV) and *in vivo* in gentamycin-exposed vestibular HCs (paper V). The signals were mainly seen in the HC nucleus. This is in line with previous studies on other cell types, which show that the activated JNK accumulates in the nucleus (Mizukami et al., 1997).

We wanted to find out whether inhibition of activation of the JNK pathway could prevent HC death. As a pharmacological tool we used a small inorganic compound, CEP-1347, which is a specific inhibitor of the JNK cascade, with no apparent effects on the parallel p38 and extracellular signal-regulated kinase (ERK) pathways (Maroney et al., 1998). More recently, the MLKs, which are located two steps upstream of JNKs in the cascade, have been shown to be the specific targets for CEP-1347 (Xu et al., 2001). CEP-1347 has been shown to be a potent inhibitor of neuronal death induced by a variety of stimuli, such as trophic factor withdrawal, oxidative stress, excitotoxicity and DNA damage (Glicksman et al., 1998; Maroney et al., 1998; Saporito et al., 1998, 1999). As shown in paper IV, treatment of cochlear explants with 100 μm neomycin for 48 hrs causes the loss of more than 90% of HCs. Coincubation of cochlear explants with neomycin and CEP-1347 provided near-total protection against HC death. In paper IV, we also showed that daily systemic delivery of CEP-1347 to noise-exposed guinea pigs provides significant attenuation of auditory HC loss, as measured by morphometric methods two weeks following the exposure. In the same individuals, protection of hearing function was demonstrated by ABR measurements, the results showing significantly less auditory threshold shift in CEP-1347-treated than in non-treated (only noise-exposed) guinea pigs. In paper V, we showed by functional tests (ABRs) and HC counts that CEP-

1347 also attenuates gentamicin-induced HC loss in guinea pigs *in vivo*. In that paper, we demonstrated that, in addition to cochlear HCs, loss of gentamicin-stressed vestibular HCs is attenuated by CEP-1347-treatment.

To give further evidence for our hypothesis that activation of JNK signaling leads to HC death, we have recently used as a model system the "knock-in" transgenic mice in which the phosphorylation sites of *c-Jun*, serines 63 and 73, are replaced by alanines (JunAA mice; Behrens et al., 1999). When cochlear explants prepared from these mutant mice were exposed to neomycin, a nearly complete HC protection was observed. Cochlear explants dissected from wildtype mice of the same strain showed a similar extent of HC loss as shown in paper IV, with most of the HCs being lost (Fig. 10, our unpublished data). Correspondingly, noise-exposed (110 dB, 8 kHz, 6 hrs) JunAA mice showed a significant protection against noise trauma, as measured by HC counts and ABR measurements (our unpublished data). Thus, these results provide direct genetic evidence that the JNK cascade regulates stress-induced HC death and they are in line with our previous data showing the protective effects of CEP-1347, the pharmacological inhibitor of JNK signaling.

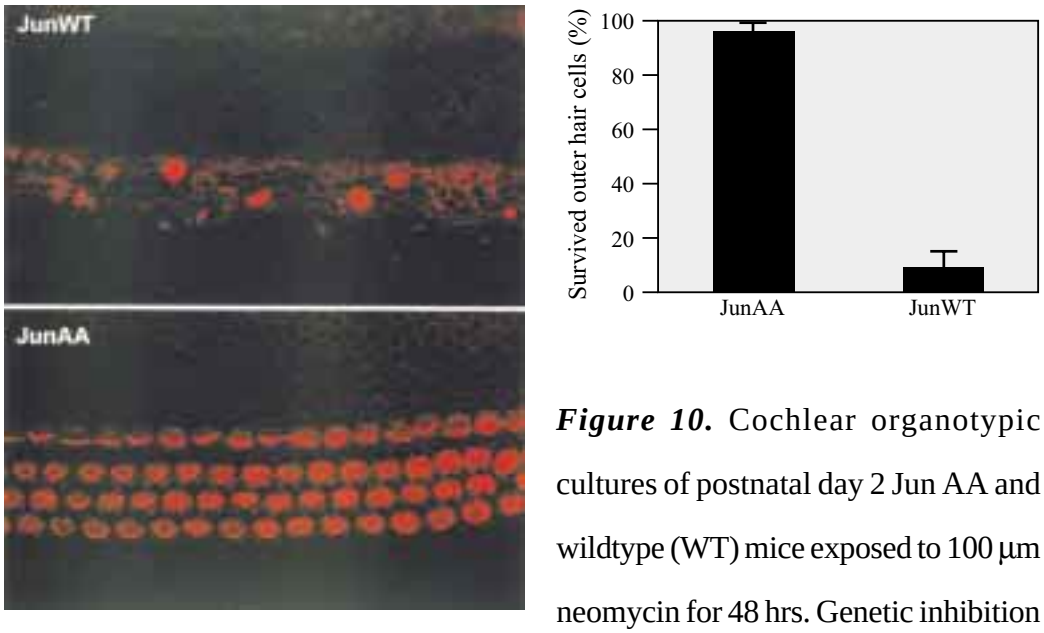

Figure 10. Cochlear organotypic cultures of postnatal day 2 Jun AA and wildtype (WT) mice exposed to 100 μm neomycin for 48 hrs. Genetic inhibition of activation of c-Jun protects against hair cell loss. Phalloidin-stained surface specimens.

Our results suggest that CEP-1347 may offer therapeutic potential in the traumatized inner ear. The i*n vitro* data show that, in addition to HC protection, CEP-1347 provides protection against the death of cochlear neurons following trophic factor withdrawal (paper IV). Thus, the JNK inhibitor might have a double-beneficial effect, acting on both sensory cell compartments, which are affected by the inner ear-specific traumas. Unfortunately, the spatiotemporal progression of HC apoptosis in response to noise or aminoglycosides has not been studied in detail. Our results indicate that the major part of HC apoptosis occurs within the few days following intense or moderate noise exposure. Therefore, if CEP-1347-based therapy is realized, its delivery as soon as possible following the insult will be important. We also believe that the possible

advantages of local delivery of CEP-1347 into the inner ear, perhaps through the round window, should be seriously investigated in the future. In addition to the traumatized inner ear, CEP-1347 (and putative other inhibitors of JNK signaling) may have therapeutic impact in preventing neuronal death associated with various neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases.

Emerging evidence implicates the involvement of JNK signaling in excitotoxin- and oxidative stress-induced apoptosis (for review, see Davis, 2000). Oxidative stress results from increased production or decreased elimination of ROS. A wealth of data suggest that oxidative stress is the underlying cause for trauma-induced HC death. Increased production of ROS has been demonstrated in the cochlea following application of ototoxic compounds and exposure to loud noise. Furthermore, impairment of the cochlea's endogenous antioxidant defense system has been shown to promote HC trauma induced by these agents. Conversely, temporarily bolstering of the antioxidant defense by exogenous compounds provides partial protection against HC loss (reviewed by Kopke et al., 1999). Oxidative stress and ROS production can induce JNK activation (Xia et al., 1995; Guyton et al., 1996, Lo et al., 1996). Furthermore, CEP-1347 has been shown to protect dopaminergic neurons against oxidative stress-induced death (Saporito et al., 1999). Taken together, we suggest that, in the traumatized inner ear, JNK signaling mediates and CEP-1347 can protect against oxidative stress-induced HC death.

4. FGF10/FGFR2(IIIb) signaling is essential for early inner ear morphogenesis (paper VI)

The FGF family consists of many structurally related proteins (to date 23 members are known) that display a variety of biological effects, including regulation of cell proliferation, migration and differentiation. There are four *Fgfr* genes, designated *Fgfr1* to *Fgfr4*, which encode transmembrane receptors with a cytoplasmic tyrosine kinase domain and an extracellular region composed of three immunoglobulin (Ig)-like domains. Alternative mRNA splicing of *Fgfrs 1-3* specifies the sequence of the carboxy-terminal half of the Ig-domain III, giving rise to the IIIb or IIIc isoform. Splice isoforms dramatically differ in ligand specificity (Ornitz et al., 1996; Fig. 11). The two isoforms often show differential expression patterns, the *IIIb* isoform being expressed mainly in epithelia and the *IIIc* variant in mesenchyme. FGFs function as short-range signaling molecules because of their high affinity to heparan sulfate proteoglycans (HSPGs) found in the ECM. HSPGs are required for efficient activation of FGFRs by FGFs (reviewed by Ornitz and Ohuchi, 2001).

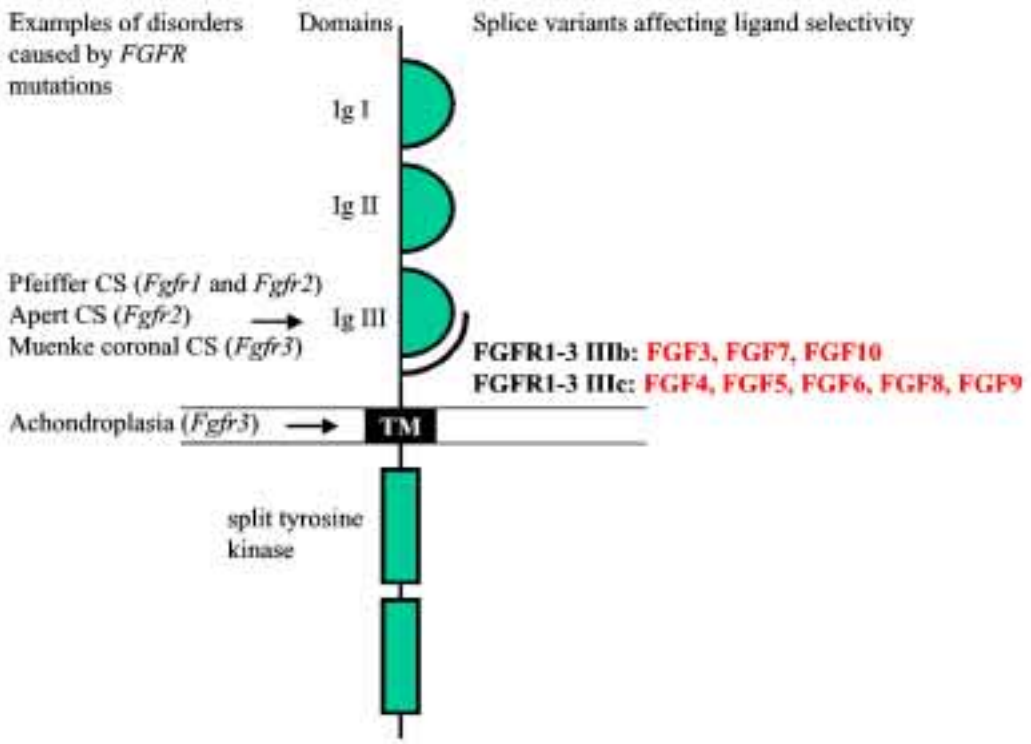

Figure 11. Schematic diagram of a prototypical FGFR protein. Three Ig-like domains are indicated by loops. Alternative splicing in the C-terminal half of the third Ig-like loop is indicated by an extra "half" loop. Also shown are some syndromes caused by *Fgfr* mutations. Their approximate locations within the protein are shown. CS, craniosynostosis.

Our initial expression studies suggested that several *Fgfs* and *Fgfrs* are expressed at the right time and place to be able to regulate both the global and fine-grained patterning of the inner ear. Due to apparent redundancy between the ligands, we thought that *Fgfr* mutations might better reveal the functional significance of FGF/FGFR signaling. Redundancy is likely to exist also between

FGFRs, but to a lesser degree than in the case of FGFs. The first knockouts generated, *Fgfr1* and *Fgfr2* null mutants, were not informative, since they die during gastrulation, prior to ear morphogenesis. More recently, isoform-specific *Fgfr* null mutants, loss-of-function *Fgfr* hypomorphs and chimeras have been generated. These transgenic strategies have made it possible to study the role of FGF/FGFR signaling during organogenesis.

Our *in situ* hybridizations detected *Fgfr2*, particularly its IIIb isoform, in the presumptive non-sensory epithelium of the otocyst. *Fgf10*, encoding the primary ligand for FGFR2(IIIb), is expressed in the sensory patches and in the neuronal compartment of the inner ear (Fig. 12). *Fgf3*, encoding another preferred ligand for FGFR2(IIIb), is also found in the sensory regions of the otocyst and, additionally, in the adjacent hindbrain rhombomeres. *Fgf3* knock out mice show severe defects in the otocyst development, albeit with reduced penetrance and variable expressivity (Mansour et al., 1993). By analyzing the phenotype of *Fgfr2(IIIb)* null mutants (DeMoerlooze et al., 2000), we concluded that FGFR2(IIIb) signaling is essential for the budding morphogenesis of the inner ear, in an analogous mode as this signaling has been suggested to regulate development of the limbs and lung (reviewed by Hogan, 1999). However, in contrast to these other organs in which FGF10/ FGFR2(IIIb) signaling is based on mesenchyme-to-epithelium interactions, in the inner ear, it operates within the epithelium, perhaps according to the boundary model as discussed above. *Fgfr2(IIIb)* null mice fail to extend sensory (cochlear duct) and non-sensory appendages from the otocyst, resulting in a severily malformed, rudimentary inner ear. Our more recent analysis of *Fgf10* knock outs (Pauley et al.,

2001) supports the suggestion that FGF10/FGFR2(IIIb) interaction regulates early inner ear morphogenesis. The inner ear phenotype of *Fgf10* null mice is somewhat milder as compared to *FGFR2(IIIb)* mutants, suggesting partial redundancy between the epithelium-derived FGF10 and FGF3 signals or an effect of hindbrain-derived FGF3 on FGFR2(IIIb)-expressing domains within the otic epithelium. This redundancy might also explain the variable phenotype seen in *Fgf3* knock outs.

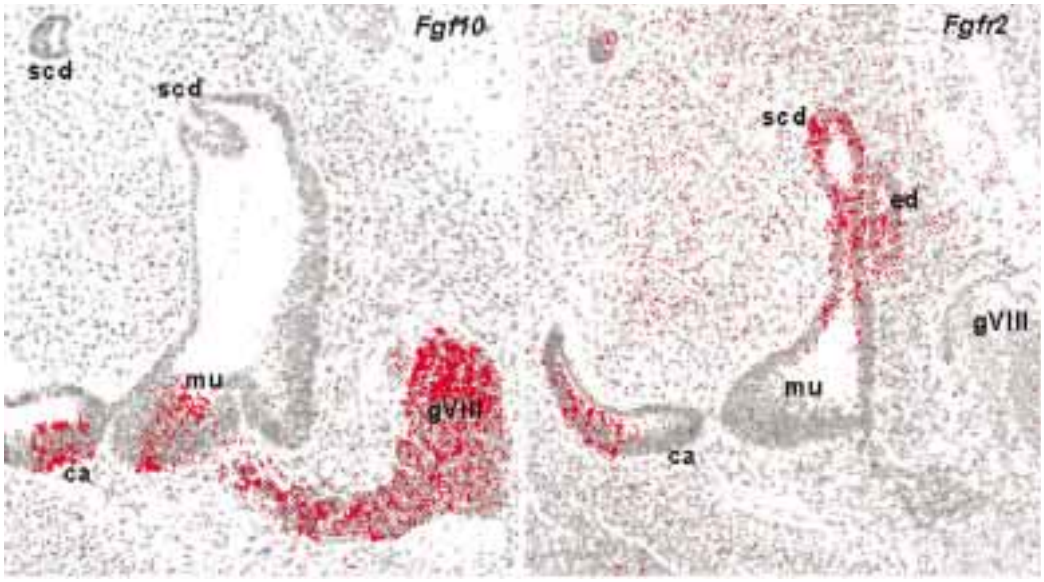

Figure 12. *Fgf10* and *Fgfr2* (pan-probe) expression in the embryonic day 13 mouse otocyst. The ligand mRNA is expressed in the sensory patches of the epithelium, in delaminating neuronal precursors and in neurons of the cochleovestibular ganglion. The receptor mRNA is expressed in a non-overlapping pattern in the non-sensory regions of the epithelium and in the surrounding mesenchyme. By using isoform-specific probes, we have found that the epithelial expression revealed by the pan-*Fgfr2* probe largely represent its *IIIb* isoform. Abbreviations: ca, crista ampullaris; mu, macula utriculi; scd, semicircular duct; ed, endolymphatic duct; gVIII, cochleovestibular ganglion.

An important next step will be to analyse the up- and downstream regulators of FGF/FGFR2(IIIb) signaling during inner ear development. Our results and the wealth of data on other organs indicate that FGF signaling is directional (crosses boundaries). For example, during the formation of the limb bud, mesenchyme-derived FGF10 binds to FGFR2(IIIb) in the overlying epithelium, inducing the formation of the apical ectodermal ridge. The ridge subsequently expresses *Fgf8*, which signals back to the underlying mesenchyme. It is likely that this kind of reciprocal loops, composed of FGFs and other signaling molecules, regulate inner ear patterning too. Concurrent with the demonstration of the critical roles of FGF/FGFR signaling during embryogenesis, several mutations in *Fgfr* genes have been elucidated as the underlying cause of various skeletal dysplasias and craniosynostosis syndromes (reviewed by DeMoerlooze and Dickson, 1997; Wilkie et al., 2001; Fig. 11). Achondroplasia is caused by mutations in *Fgfr3*, while mutations involving notably *Fgfr1* and *Fgfr2* cause craniosynostosis syndromes such as Pfeiffer, Apert, Crouzon, Muenke, and Jackson-Weiss syndromes. Deafness has been documented in patients with craniosynostosis syndromes (Muenke et al., 1997). However, no thorough analysis has been performed regarding the putative linkage between hearing loss (sensorineural or conductive) and *Fgfr* mutations.

5. FGFR1 is essential for the generation of the organ of Corti (paper VII)

Initially, we found *Fgfr1* expression at several stages of inner ear development, specifically in the regions contributing to the formation of the OC. To reveal the functional significance of FGFR1, we have analysed the inner ear phenotype of various

Fgfr1 loss-of-function mutant mice, including two hypomorphic lines in which 80% and 90% of the gene activity is disrupted (Partanen et al., 1998). To obtain further evidence of the role of *Fgfr1* on the inner ear development and knowing that *Fgfr1* knock outs die during early-embryogenesis, we targeted *Fgfr1* null mutation to distinct head structures, including the otic epithelium, by Cre-loxP technology. We used a mouse line carrying a conditional *Fgfr1* allele, the *flox* allele, which can be inactivated by Cre recombinase. These mice were crossed with the *Foxg1-Cre* mouse line in which the *Cre* coding sequence is targeted to the *Foxg1* locus (Hébert and Connell, 2001). This mating generates embryos in which *Fgfr1* is inactivated only in regions where *Foxg1* is expressed. *Foxg1* is a winged-helix transcription factor that is expressed in the otic epithelium from the earliest stages onward (Hébert and Connell, 2001).

We found an interesting cochlear phenotype in *Fgfr1* loss-of-function mutants. A dose-dependent disruption of the organ of Corti was found, full inactivation causing the loss of 85% of differentiating HCs, as analysed at birth. The remaining HCs and supporting cells were arranged in distinct patches rather than being distributed in a scattered manner. (Fig. 13). We found that the main cause of the reduced numbers of HCs and supporting cells is the dramatically reduced proliferation of early precursors of the organ of Corti. In addition, our results suggest that FGFR1 may have a role later role during cytodifferentiation within the organ of Corti. We believe that our results showing the prominent stimulatory effect of FGF/FGFR1 signaling on precursor-cell proliferation might

be useful in the future in establishing ways how to activate and/or expand the precursor-cell pool that could give rise to new HC, even in the adult hearing organ.

Figure 13. Phalloidin-stained cochlear surface specimen of a *Fgfr1*$^{n15YF/n15YF}$ hypomorphic mouse at birth. In contrast to the four continuous hair cell rows seen in normal cochleas, there is sensory-cell loss in the cochleas of the hypomorphs and the remaining cells are arranged in distinct patches.

CONCLUSIONS

1.The neurotrophins BDNF and NT-3 are essential for the establishment of afferent innervation of the rat inner ear.

2. BDNF/TrkB interaction is involved in the innervation of regenerated HCs in adult chicks. Many aspects of the reinnervation process recapitulate embryonic innervation.

3. JNK cascade mediates apoptotic HC death following noise- and ototoxic drug-induced traumas. This death can be attenuated *in vitro* and *in vivo* by a specific inhibitor, CEP-1347.

4. FGFR2(IIIb) is essential for early inner ear morphogenesis.

5. FGFR1 is essential for the formation of the organ of Corti.

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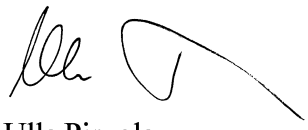

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