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Zebrafish (*Danio rerio*) neuromast: Promising biological endpoint linking developmental and toxicological studies

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ABSTRACT

Aquatic toxicology is facing the challenge to assess the impact of complex mixtures of compounds on diverse biological endpoints. So far, ecotoxicology focuses mainly on apical endpoints such as growth, lethality and reproduction, but does not consider sublethal toxic effects that may indirectly cause ecological effects. One such sublethal effect is toxicant-induced impairment of neurosensory functions which will affect important behavioural traits of exposed organisms. Here, we critically review the mechanosensory lateral line (LL) system of zebrafish as a model to screen for chemical effects on neurosensory function of fish in particular and vertebrates in general. The LL system consists of so-called neuromasts, composed of centrally located sensory hair cells, and surrounding supporting cells. The function of neuromasts is the detection of water movements that is essential for the fish's ability to detect prey, to escape predator, to socially interact or to show rheotactic behaviour. Recent advances in the study of these organs provided researchers with a broad area of molecular tools for easy and rapid detection of neuromasts dysfunction and/or disturbed development. Further, genes involved in neuromasts differentiation have been identified using auditory/mechanosensory mutants and morphants. A number of environmental toxicants including metals and pharmaceuticals have been shown to affect neuromasts development and/or function. The use of the LL organ for toxicological studies offers the advantage to integrate the available profound knowledge on developmental biology of the neuromasts with the study of chemical toxicity. This combination may provide a powerful tool in environmental risk assessment.

1. Introduction

The aquatic resources are severely affected by pollution, since human population keeps growing concomitant with the ever increasing use and release of chemicals into the environment (Schwarzenbach et al., 2006). Ecotoxicology helped to reduce the risk of acute effects caused by the release of high volumes of industrial chemicals. The subtle and chronic effects caused by specifically acting compounds, derived for example from personal care products or pharmaceuticals, are not yet adequately addressed by the standard toxicological tests (Segner, 2007; Eggen et al., 2004; Cunningham et al., 2006). These compounds, occurring at low concentration and in mixtures, affect subtle physiological traits in organisms and may directly or indirectly cause long-term adverse ecological effects (Scott and Sloman, 2004).

Examples of these compounds are pharmaceuticals and heavy metals that are known to affect the sensory system of humans as well as of fish. Many pharmaceuticals have been found in sewage treatment plant and river water at concentrations up to several μg/L (Hirsch et al., 1999). When pharmaceuticals are administered to patients, some of their active ingredients may not be completely metabolised (Anderson et al., 2004; Schwab et al., 2005; Kolpin et al., 2002). In a recent study it was described that 50% of the drugs found in the waste water are in the original bioactive form, and 50% in a metabolized form (Lienert et al., 2007). Since pharmaceuticals have been designed to be active, it is to be expected that these compounds and their metabolites will have similar effects in the aquatic environment and may also have toxic effects on non-target organisms. These compounds and their metabolites are only partially removed by actual waste water treatment plants, and hence they are, though at low concentrations, found in the environment. A well-studied example of a drug entering the aquatic environment

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and having effects on aquatic organisms is the synthetic hormone 17β-ethinylestradiol, which has been shown to have endocrine disrupting effects in wildlife (Nash et al., 2004; Fenske et al., 2005). In addition, human-related activities, such as industrial and consumer waste, or acidic rain, release heavy metals, which contaminate streams, lakes, rivers, and groundwater. Metal pollution is a serious concern for aquatic wildlife as these inorganic compounds tend to accumulate in biological organisms.

While for the risk assessment of acute exposures to non-specifically acting chemicals ecotoxicology has developed a valuable set of toxicological tools, the assessment of low-dose, chronic effects of more specifically acting compounds ask for novel, more mechanisms-oriented tools (Eggen and Segner, 2003; Eggen et al., 2004; Hutchinson et al., 2006). More emphasis has to be placed on the toxicant-induced impairment of physiological functions that does not directly lead to lethality but still significantly diminishes the ability of organisms to cope with their environment (Relyea and Diecks, 2008). One such function is the sensory system, which is essential for behaviour, social interactions, prey detection and predator avoidance. Toxicant-induced impairments of sensory functions have therefore an immediate ecological relevance. In fact, it has been shown that a variety of chemicals are able to alter sensory function and structure (Hansen et al., 1999; Bettini et al., 2006; Ottinger et al., 2008). In humans, some pharmaceuticals were shown to affect hearing and balance disorders, and heavy metals affect the vision, taste, olfaction and auditory functions (Gobba, 2003; Loeffler and Ternes, 2003). These chemicals reach the environment and affect similarly the aquatic organisms (Loeffler and Ternes, 2003). In fish, aminoglycoside antibiotics and heavy metals have been shown to affect the olfactory and mechanosensory systems (Hansen et al., 1999). The latter system is closely related to the hearing system of higher vertebrates. Beside possessing the typical vertebrate inner ears with both hearing and vestibular organs, all fish possess additional structures that contain sensory hair cells, the lateral line (LL) organs (reviewed by Popper, 2000). The LL is composed of rosette-like structures called neuromasts, located on the surface of the animal and readily accessible to analysis. The neuromasts on the head form the so-called anterior LL system (ALL). The posterior LL (PLL) consists of the neuromasts of the trunk and tail. Mature neuromasts are composed of hair cells and supporting cells (Ghysen and Dambly-Chaudiere, 2007, 2004). The LL hair cells are supposed to gain their mechanotransduction capacity at 3–4 days post-hatching (dph) as it is at this time when they become innervated (Raible and Kruse, 2000), and when larval behaviours become consistent with possessing a functional LL (Nicolson et al., 1998). The function of the mechanosensory LL is mainly to allow the fish to orient relative to a water current (rheotaxis), to hold a stationary position in a stream, to detect prey, or to avoid predators.

The LL has been particularly well studied in zebrafish, an excellent model organism frequently used in (eco)toxicology (Segner, 2009; Hill et al., 2005; Scholz et al., 2008), pharmacological screening (Zon and Peterson, 2005; Goldsmith, 2004) and neurotoxicology (Linney et al., 2004). Zebrafish embryos enable small-scale and high-throughput analyses. Moreover, besides looking at acute toxicity, fish embryos are also suitable for detecting possible adverse long-term effects. The zebrafish LL is a favourable system to explore gene function and effects of contaminants, given its simplicity, resolution, sensitivity to toxicants and accessibility.

The zebrafish LL analysis offers many advantages, particularly when compared to cell lines studies, where it is difficult to mimic *in vivo* conditions (Stone et al., 1996; McFadden et al., 2003). Indeed, cell-based assays do not allow assessment of the complex metabolism that causes toxicity in animals (Stone et al., 1996; McFadden et al., 2003). For example, cultured cochlear epithelia lack supporting cells which also play a role in hair cell functions (Ton and Parng, 2005). Neuromasts have been shown to be an excellent system for studying ototoxicity (damage of the ear), as their peripheral sensory neurons are in direct contact with the surrounding water containing pollutants. Another advantage is that hair cells can be easily stained by various fluorescent dyes without complicated histological preparation even in the living larva (Collazo et al., 1994; Seiler and Nicolson, 1999; Hernandez et al., 2006a). The level of hair cell staining can be imaged and quantified using morphometric analysis, and the effects can be assessed for the screening of potential ototoxic pollutants (Ton and Parng, 2005). Moreover, the effects of contaminants can also be assessed on the supporting cells, which are not accessible at all in *in vitro* studies.

This review summarizes the efforts to elucidate the normal development of neuromasts and its disruption by toxicants that have been reported so far (Fig. 1). The parallels drawn between blocking LL development through gene knock down or through chemical treatment show that this organ might provide a powerful tool to combine developmental and toxicological analyses. In fact,

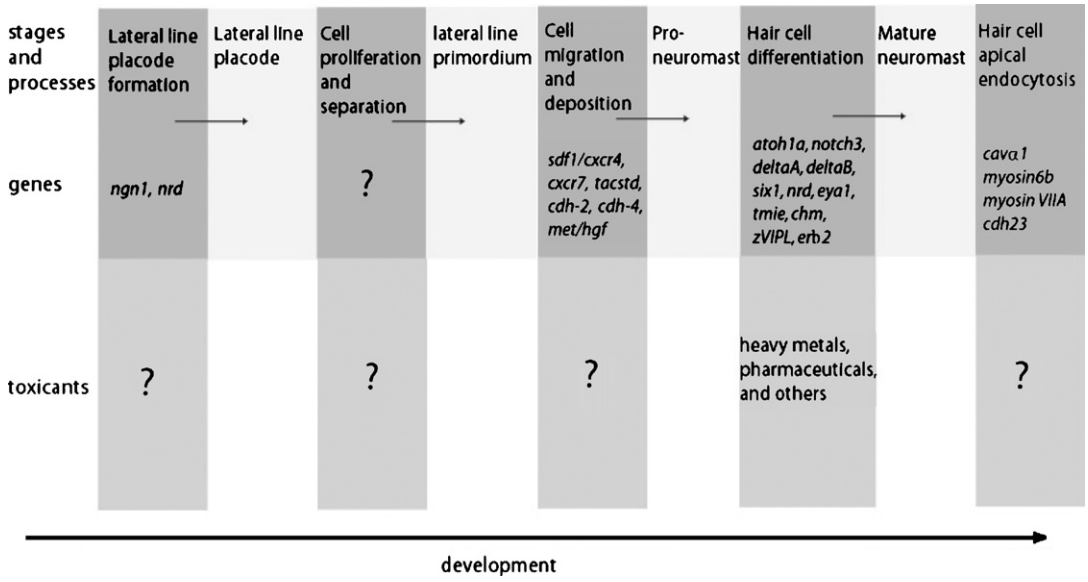

Fig. 1. Schematic drawing linking the developmental studies and the toxicological studies performed on neuromasts.

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Table 1
List of gene function studies in zebrafish (*Danio rerio*) LL.

Function in LL	Mutants/morphants	Genes	Detection methods	References
Placode formation	*ngn1* mutant/morphant	*ngn1*	IHC anti-HU, DiAsp, TO-PRO3	Andermann et al. (2002)
		nrd	Anti-acetylated tubulin, glial marker 6D2, IHC anti-Cld, anti-phosphohistone H3	Lopez-Schier et al. (2004)
Primordium migration and pro-neuromast deposition	*sdf1*/*cxcr4* morphants, *cxcr7* morphant	*sdf1*, *cxcr4*, *cxcr7*	DiAsp, ISH with Nodal inducible RNAs	David et al. (2002); Valentin et al. (2007)
			cldnbGFP transgenic line, Rhodamine dextran	Haas and Gilmour (2006)
	cdh2 morphant, *glass onion* mutant	*cdh2*	DASPEI, IHC anti-HU, anti-acetylated tubulin	Kerstetter et al. (2004)
	cdh4 morphant	*cdh4*	DASPEI, IHC anti-HU and zn5, anti-acetylated tubulin	Wilson et al. (2007)
	tacstd morphant	*tacstd*	DiAsp, alkaline phosphatase labelling	Villablanca et al. (2006)
	met/hgf morphant	*met/hgf*	DASPEI	Haines et al. (2004)
Hair cell differentiation	*mib* mutant	*atoh1a, notch3, deltaA, deltaB*	ISH with *atoh1a, notch3, delatA, deltaB*, IHC anti-actetylated tubulin	Itoh and Chitnis (2001)
	mib mutant	*six1*	ISH with *six1*	Bessarab et al. (2004)
	mib mutant, *atoh1a, nrd* morphants	*atoh1a, nrd*	ET4, ET20 transgenic line, DiAsp, IHC anti-actetylated tubulin	Sarrazin et al. (2006)
	dog-eared mutant, *eya1* morpant	*eya1* *eya1*	DASPEI, ISH *eya1*	Kozlowski et al. (2005)
	tmie morphant	*tmie*	DASPEI, ISH with *cxcr4b, atoh1a*	Shen et al. (2008)
	ru848 mutant	*chm*	DiAsp, SEM	Starr et al. (2004)
	hi472 mutants	*zVIPL*	IHC with anti-ClaudinB, anti-acetylated Tubulin, DiAsp, acridine orange	Chong et al. (2008)
	$er\beta_2$ morphants	$er\beta_2$	FM1–43, ISH with *cld, k15*, ET4	Froehlicher et al. (2009)
	skylab mutant	–	FM1–43	Nicolson et al. (1998)
Hair cell apical surface endocytosis	*Cav1a* morphants	*Cav1a*	DASPEI, SEM	Nixon et al. (2007)
	ru920 mutant	*myosin6b*	DiASp, TO-PRO-3,	Kappler et al. (2004)
	myo6b morphant	*myosin6b*	IHC anti-parvalbumin3, anti-3A10	
	mariner mutant	*myosin VIIA*	Oregon green phalloidin	Ernest et al. (2000)
	sputnik	*cdh23*	IHC anti-phalloidin, TEM	Sollner et al. (2004)
	orbiter, mercury, gemini	–	FM1–43	Nicolson et al. (1998)
	astronaut, cosmonaut mutant	–		

the more molecular understanding we get of the normal developmental process of neuromasts formation, the better tools can be made available to assess and understand toxic effects of pollutants on this sensory system.

2. Gene function studies in neuromasts

In order to be able to evaluate the effects of pollutants on the LL system, it is important to understand the molecular basis of its development. In this part of the review, we will focus on the research performed in zebrafish that can link genetic expression with a function in the development of the LL. Many genes involved in the normal development of the sensory LL, have been identified and functionally characterized (Table 1). The knowledge in this area increased very fast in recent years mainly because of the availability of several mechanosensory mutants, as well as of a simple reverse genetic technique, the morpholinos (MO) knock down technology. Importantly, the development of the LL can be easily blocked, and the consequences of disrupted neuromast development can easily be detected on the physiological and behavioural level, so that molecular changes can be phenotypically anchored. The following paragraphs are structured around the genes that play a role in placode formation, primordium migration, pro-neuromast deposition, hair cell differentiation and hair cell apical surface endocytosis.

2.1. Genes involved in placode formation

Placodes arise from the neural ectoderm in a specific region of the neural plate during early neurogenesis. In response to specific molecular signals, the different cephalic placodes are induced and later develop into diverse sensory organs. The otic placode forms the inner ear and the LL placode forms the LL organs. With the help of different mutants and morphants, the role of genes involved in LL placode formation has been investigated in zebrafish.

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2.1.1. ngn1 mutants and morphants

The genes *neurogenin* (*ngn1*) and *neuroD* (*nrd*) are considered to be the molecular markers for cranial neurogenic placodes in zebrafish. The *ngn1* gene is expressed in neurogenic placodes, including the LL placodes, and in cells within the otic vesicle (Andermann et al., 2002). The otic vesicle generates the sensory hair cells in the inner ear, while the LL placodes give rise to sensory ganglia and to migrating primordia that deposit neuromasts along the head and body (Gompel et al., 2001).

In *ngn1* morphants, the differentiation of all cranial ganglia is blocked, as was shown with specific markers of neuronal cell bodies (anti-HU antibody) and of neurogenic placodes (*nrd*); cranial ganglion neurons were absent and *nrd* expression was downregulated in neurogenic placodes. The latter observation suggested that *nrd* acts downstream of *ngn1* in the development of LL placodes (Andermann et al., 2002). The morphants were also touch insensitive, suggesting a role of *ngn1* in the formation of trunk sensory neurons. The formation or migration of the LL primordium was not affected in the *ngn1* morphants, as was shown by the presence of *eyes absent-1* (*eya1*) expression, a primordium marker, and by the presence of neuromasts at the tip of the tail, as well as differentiated hair cells (Andermann et al., 2002). This study showed that LL neuromasts, derived from migrating LL primordia, can differentiate and persist for several days even in the absence of differentiated LL ganglia. In addition, interactions with the nerve were not necessary for neuromasts differentiation. This suggests that placode-derived neuromast precursors develop in the absence of a functional *ngn1* gene, and thus that the two derivatives of the LL placodes, the ganglia and the migrating primordia, are under separate genetic control.

These results have been confirmed by the study of Lopez-Schier et al. (2004), where the formation of supernumerary neuromasts between neuromast of normal larvae has been shown in *ngn1* morphants and mutants. The presence of these intercalary neuromasts could not be explained by an increase in primordial cells or by higher mitotical activity of primordia. Also at 48 h post-fertilization (hpf) no difference in the number of neuromasts was found, suggesting that the supernumerary neuromasts in *ngn1* mutants do not originate during the initial development of the LL. The extra neuromasts formed were finally attributed to the absence of glial, supported by phenotype comparison with *colourless* mutants that show similar interneuromasts and are characterized by the absence of the *sox10* gene coding for a transcription factor associated with the development of nonectomesenchymal derivatives of the neural crest. The absence of *sox10* leads to defective development of peripheral glia but does not affect the development of sensory axons innervating the LL (Lopez-Schier et al., 2004). This research confirmed that the *ngn1* gene is not necessary for the migration of the primordium and the initial development of neuromasts, but is needed for the generation of the sensory ganglia from the placode. The intercalary neuromasts phenotype observed in the *ngn1* morphant fish is due to the absence of the glial cells that normally accompany the migrating LL nerve. This lack of glia obviously inhibits the assembly of interneuromast cells into neuromasts (Lopez-Schier et al., 2004).

Using these mutants/morphants and the knowledge gained in the process of placode formation, one may analyse the effects of chemicals on these developmental processes.

2.2. Genes involved in primordium migration and pro-neuromast deposition

The PLL ganglion is formed from the differentiated LL placode and contains a large compartment of around 100 cells, the so-called PLL primordium. At 20 hpf the primordium begins to migrate and neuromast deposition occurs. During this migration, clusters of cells are deposited, the so-called pro-neuromasts (Sapede et al., 2002). The LL emerged as a favourable system for analysing the genetics of cell migration and its control.

2.2.1. sdf1 and cxcr4 morphants

The CXC chemokine receptor (CXCR4) and its ligand, stromal-derived-factor-1 (SDF1), have been shown to drive the migration of the primordium and to define its stereotyped migration route (David et al., 2002). After knocking down of either the ligand or the receptor, the primordium was found to move little or not at all at 32 hpf. At 52 hpf, when the PLL normally comprises a line of 7–8 neuromasts, the *sdf1a* morphants showed no or very few neuromasts along the trunk. However, the neuromasts of the head developed normally. Thus, *sdf1a* is an essential component regulating migration of the PLL primordium. The inactivation of *cxcr4b* at 3 dpf also resulted in major defects in the PLL, while the ALL developed normally (David et al., 2002). The authors concluded that the migration of the PLL primordium is predominantly regulated by the interaction between the SDF1 ligand, which determines the path, and its CXCR4 receptor, which controls the movement. Further research revealed that also other receptors are involved in the primordium migration. In a recent loss-of-function study it was shown that the CXCR4 is only needed for the cells at the very tip of the tail (Haas and Gilmour, 2006), while in the trailing region CXCR7 seems to be necessary for primordium migration (Valentin et al., 2007).

2.2.2. cadherins morphants and mutants

Cadherins, a type of cell surface molecules, have been shown to play a role in cell adhesion. Different members of the cadherin family have been found in zebrafish neuromast, namely *cadherin-2* (*cdh2*), *cadherin-4* (*cdh4*) and *cadherin-23* (*cdh23*). *Cdh2* has been found to be expressed in developing cranial ganglia, in the LL ganglia and in neuromasts. The LL system did not develop normally after knock down of *cdh2* (Kerstetter et al., 2004). The morphants had a reduced number of neuromasts on the head, trunk and tail, some neuromasts were located too close to each other. The exact mechanisms underlying the disrupted processes are not known. Additional data from *cdh4* morphants revealed that the cranial and LL ganglia were disorganized, appeared smaller and altered in shape compared to control embryos. Shorter LL nerves and a reduced number of neuromasts were also observed, suggesting a disrupted migration of the LL primordium after the knock down of cadherins (Wilson et al., 2007). *Sputnik* mutant showed mechanosensory defects caused by the *cdh23* mutation. This study showed a role of *cdh23* in the development and signal transduction of the extremity of hair bundles (see Section 2.4) (Sollner et al., 2004). It needs yet to be verified whether cadherins are important for primordium migration or for hair cell development, or for both processes.

2.2.3. tacstd morphants

The tumour-associated calcium signal transducer gene (*tacstd*) codes for cell surface glycoproteins and is postulated to have a function in cell adhesion, tumorigenesis and regulation of proliferation in mammalian cells (Went et al., 2004). In zebrafish, the *tacstd* gene encodes a membrane protein that is homologous to the TACSTD1/2 mammalian proteins (Villablanca et al., 2006). A part of the molecular machinery involved in primordium migration is shared with that of invasive tumour cells. TACSTD expression was found in ALL and PLL neuromasts. At 28 hpf, expression is maintained in the migrating PLL primordium as well as in pro-neuromasts, and later it is observed in the mature neuromasts. Within the pro-neuromasts and mature neuromasts, the *tacstd* expression pattern appears ring-like, suggesting specific expression in the supporting cells (Villablanca et al., 2006). Comparison of *tacstd* expression with that of the proneural gene, the *atonal homologue* (*atoh1a*), allowed concluding that both are expressed in largely complementary

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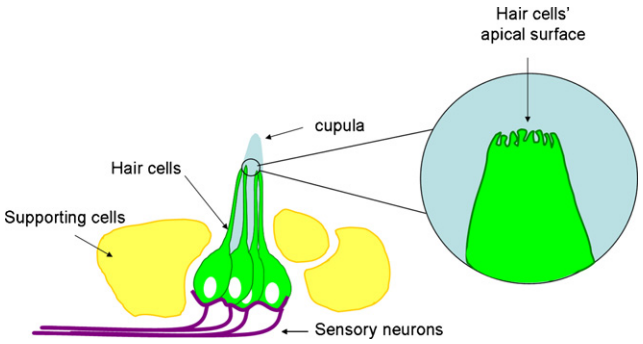

Fig. 2. Schematic drawing of the supporting and hair cells connected with the sensory neurons. The hair cell surface is represented with the apical membrane's numerous invaginations.

patterns in pro-neuromasts. It was discovered by a loss-of-function analysis that *tacstd* is necessary for neuromast development. A significant reduction in number, or even absence of neuromasts was observed in *tacstd* morphants. As one or two terminal neuromasts at the tip of the tail could still be found in some morphants, the authors concluded that *tacstd* is not involved in primordium migration, but rather plays a role in cell deposition. This is confirmed by the presence of *tacstd* in both ALL and PLL neuromasts, suggesting that *tacstd* is not involved in the control of CXCR4-dependent migration, as CXCR4 drives exclusively the PLL primordium and not the ALL (David et al., 2002).

2.2.4. *met* morphants

Receptor tyrosine kinase (Met), a high affinity cell surface receptor, and its ligand, hepatocyte growth factor (Hgf), are key regulators of the normal cellular processes as well as of the development and progression of many cancer types. In zebrafish, Met and Hgf are required for the correct morphogenesis of the hypaxial muscles in which *met* transcripts have been detected (Haines et al., 2004). Met signalling showed to be needed for pro-neuromast deposition by the PLL primordium. Met is present during the entire period of primordia migration, but it is not expressed within deposited pro-neuromasts or neuromast clusters. *Met* morphants showed a reduction in the number of deposited neuromast clusters stained with a fluorescent dye. The number of hair cells within the deposited neuromast was also reduced. With the supporting cell marker gene, *follistatin*, a lack of supporting cells was also revealed. The authors identified that *met* plays a role in neuromast deposition, but not in the correct migration of the PLL primordia along the body axis, as shown with a non-affected primordia marker gene, *prox1* (Haines et al., 2004).

Altogether, the primordium migration is determined by the interaction between the SDF1, CXCR4 and CXCR7. Two molecule types, which are essential for cell adhesion, have also shown to be important for neuromast development: the cadherins, which are suspected to have a role in cell migration, and the TACSTD which together with Met is needed for neuromast deposition.

The knowledge of the mechanisms of primordium migration and pro-neuromast deposition could help to decipher the effects of chemicals on this stage of neuromast development.

2.3. Genes involved in hair cell differentiation

After cell deposition, the neuromast maturation is initiated. The cells are differentiating and a rosette shape structure is formed with the supporting cells in the periphery (Fig. 2). In the center, the cells differentiate into hair cells with stereocilia and kinocilium. The *mind bumb* mutants, together with other mutants and morphants, helped to decipher the process of hair cell differentiation.

2.3.1. *mind bomb (mib)* mutants

The *mib* mutant has been discovered by searching for neurogenic phenotypes in the Boston zebrafish mutagenesis screen (Haddon et al., 1998a). The zebrafish *mib* mutants carry a mutation in the *mib* gene, which encodes a RING E3 ligase required for Notch activation (Itoh et al., 2003; Whitfield, 2005). A failure of lateral inhibition mediated by Notch signalling is observed in these mutants (Itoh and Chitnis, 2001; Itoh et al., 2003; Haddon et al., 1998b). The phenotype of this mutant, characterized by supernumerary hair cells, (Jiang et al., 1996; Schier et al., 1996), was used to analyse the different roles of proneural genes in hair cell differentiation.

Notch/Delta signalling is known to play a role in cell fate decision in hair cells of the mammalian inner ear (Hawkins et al., 2007). A similar mechanism is involved in hair cell differentiation in zebrafish neuromasts. The normal expression patterns of *notch2*, *deltaA*, *deltaB*, and *atoh1a* were analysed and compared with those in the *mib* mutant (Itoh and Chitnis, 2001). The expression of *notch3* in the *mib* mutants was down-regulated in the primordium of the LL, suggesting that it is excluded from selected hair cells in the maturing neuromasts. The three other proneural genes showed to be up-regulated in the hair cells of the neuromast primordium. The *atoh1a* gene expression was not restricted to specific cells, thus causing the formation of too many hair cells at the cost of supporting cells. This correlates with a corresponding increase in the number of cells expressing *deltaA* and *deltaB* (Itoh and Chitnis, 2001). Furthermore, a recent study showed a role of the different members of the Notch signalling pathway in the hair cell regeneration process. Hair cells were able to regenerate after neomycin damage. The renewal of hair cells was accompanied by an increase in supporting cell proliferation and by a *notch3, deltaA* and *atoh1a* up-regulation (Ma et al., 2008).

In addition to the *atoh1a* up-regulation, *nrd* also showed to be up-regulated in hair cell precursors of the *mib* mutant. The loss-of-function analysis of both genes revealed that *atoh1a* and *nrd* are essential for hair cell development, but not for other cell types in the neuromasts (Sarrazin et al., 2006). The phenotype produced by the loss of *atoh1a* was rescued by injection of *nrd* mRNA. This suggested that in the hair cell context *nrd* is regulated by *atoh1a*, whereas in sensory neurons *nrd* is regulated by *ngn1*.

Also *six1* expression was investigated in the *mib* mutant (Bessarab et al., 2004). The *six* gene family is known to be involved in morphogenesis, organogenesis, and cell differentiation. The expression of *six1* at 12 hpf was observed in the region that gives rise later to the otic vesicle as well as ALL and PLL placodes. At 24 hpf, *six1* was mostly expressed in the otic placodes, LL placode, vestibular ganglia and somites. In neuromasts of the midbody LL, the *six1* expression was detected first at 48 hpf in wildtype, reaching its peak at 72 hpf with stronger staining at the basal region of the neuromast, where bodies of hair cells are localized. In the *mib* mutant, *six1* expression was elevated at 48 hpf in the neuromasts. In addition, the excessive and premature production of hair cells was seen in the sensory patches of the *mib* inner ear (Haddon et al., 1998a). These results suggested that, during sensory cells differentiation in the inner ear and LL, *six1* is regulated by the Notch pathway (Bessarab et al., 2004).

2.3.2. *dog-eared mutants (-eya1)*

The zebrafish mutant *dog-eared* is characterized by abnormal morphology of the inner ear and LL sensory system (Whitfield et al., 1996). The *dog-eared* embryos are less responsive to vibrational stimuli, fail to maintain balance when swimming, and may circle when disturbed, a characteristic of fish with vestibular defects (Nicolson et al., 1998). In the developing otic vesicle and migrating primodium of the PLL, the homozygous *dog-eared* embryos showed cell death in abnormal places and in increased levels. The *dog-eared* locus encodes the *eya1* gene. It is known that *e* ya1 is required for cell

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differentiation and survival in the fly eye (Bonini et al., 1993). The *eya1* gene is expressed during embryogenesis in cells of the anterior pituitary, olfactory, otic, and LL placodes, as well as in the somites, branchial arches and pectoral fins of zebrafish (Sahly et al., 1999). A recent study showed that *eya1* is required for survival of cells in the otic vesicle, including those cells that will differentiate into hair cells (Kozlowski et al., 2005). Comparison of the *dog-eared* mutants with the *eya1* MO injected embryos showed that both phenotypes are similar, suggesting that *eya1* is required for cell survival in the otic vesicle, including the cells that will differentiate into hair cells.

2.3.3. *tmie* morphants

In mice and humans, a transmembrane inner ear gene (*tmie*) mutation causes vestibular dysfunction and profound hearing loss. In mice, *tmie* is expressed in the inner ear, brain, liver, kidney and lung. In zebrafish, the expression of *tmie* was found in the brain and the inner ear (Shen et al., 2008). After knocking down *tmie* expression, the morphants showed abnormalities in swimming behaviour. Most morphants swam in circles and exhibited altered swimming after touch stimuli or dish swirling, even at 6 days post-fertilization (dpf). The morphants also lost their balance control. Further investigation on the development of the inner ear and LL system using a fluorescent live staining revealed that morphants had fewer active hair cells than control fish (Shen et al., 2008). The expression of *cxcr4b*, a primordium marker, was used to show that the lack of hair cell activity is not a result of delayed migration of the LL primordium. The expression of *atoh1a* showed that the hair cell differentiation occurs soon after the deposition of pro-neuromasts, indicating that the early differentiation of the hair cells was not affected by *tmie* knock down. However, the delay in the maturation and/or the activity of the hair cells suggests that *tmie* is participating in maturation, function and possibly maintenance of the hair cells in the inner ear and LL organs.

2.3.4. *ru848 mutants (-chm)*

After a mutagenic screen performed to find proteins important for hair cell differentiation, larvae showing inner ear and LL organ development and function defects, causing acoustic and balance disorders, were selected, and the phenotypes were further analysed. It was discovered that the mutant *ru848* lacks 90% of the hair cells at 5 dpf (Starr et al., 2004). The authors identified the mutated gene to be the *choroideremia* (*chm*) gene and confirmed its role in hair cell development using the knock down technique. This gene encodes the Rab escort protein 1, known to play a role in many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, as well as membrane fusion. Using live staining and scanning electron microscopy (SEM), they could identify that in *chm* morphants the periderm cells appeared relatively normal, but in the center of the neuromast less kinocilia were detected and were much shorter than those seen in the wildtype (Starr et al., 2004). The retina was also affected by this mutation.

2.3.5. *hi472 mutants (-zVIPL)*

A retroviral insertional mutagenesis screen showed that the *hi472* mutation, caused by a retroviral insertion into the vesicular integral protein-like gene (*zVIPL*), resulted in a reduction of mechanosensitivity, indicated by a loss of escape behaviour (Chong et al., 2008). The *hi472* mutant suffers from a severe loss of hair cells in the neuromasts, as well as a reduction in supporting cells. The *zVIPL* mutation affected the Delta-Notch signalling, leading to an increase of *notch* expression. The phenotype could be partially rescued by treatment with an inhibitor of Notch signalling. Therefore, *zVIPL* is a necessary component of Delta-Notch signalling during neuromast development in the LL of zebrafish.

2.3.6. *erβ_2 morphants*

The presence of hormone receptors in the sensory hair cells has been examined in our own laboratory. Recently, estrogen receptor β_2 (*er*β_2) mRNA has been shown to be expressed in supporting and hair cells of zebrafish LL (Tingaud-Sequeira et al., 2004). After knock down, the larvae showed a circling swimming behaviour, typical for mechanosensory mutants. Staining with a vital dye showed that the neuromasts of the morphants lacked functional hair cells. Using cellular markers for supporting cells (*claudinb* and *keratin15*), a normal migration of the primordium and deposition of the supporting cells was observed. However, after injecting the *er* β_2 MO in ET4 transgenic fish expressing green fluorescent protein (GFP) in the hair cells (Parinov et al., 2004), a lack of hair cell development was observed, suggesting a role of *er* β_2 in hair cell differentiation. Using microarrays, an up-regulation of the *notch1a* and *notch3* gene was measured, also confirmed by ISH in the supporting cells of the neuromast (Froehlicher et al., 2009). These data suggest an interaction between the estrogen and the Notch signalling pathways during hair cell differentiation in the LL.

In conclusion, besides the proneural genes such as *deltaA, deltaB, atoh1a, nrd, six1*, several other genes revealed to be essential for hair cell development in the LL of zebrafish. The gene *eya1* showed to be essential for hair cell survival, *tmie* was suggested to be involved in hair cell maturation, function or maintenance, *chm* showed to be needed for the presence of normal kinocilia, *zVIPL* for the supporting and hair cell development and *er*β_2 for hair cell differentiation.

Chemicals that can affect hair cell differentiation could be screened for, using the mutants and morphants described above.

2.4. Genes involved in hair cell apical surface endocytosis

Sensory hair cells need a fast rate of synaptic release in order to transmit the mechanosensory signals to the neuronal system. This is ensured by the surface of hair cells that is pitted with many invaginations of the apical membrane (Fig. 2). This membrane is thought to have a high turnover rate, with a permanent replacement of old proteins by freshly synthesised proteins. The release and uptake of synaptic transmitter and the renewal of membrane proteins, such as ion channel proteins, are guaranteed by the mechanism of endocytosis. Different pathways are known to play a role during endocytosis, including clathrin-dependent and caveolin-dependent pathways.

2.4.1. *cav1αmorphants*

Caveolins are integral membrane proteins contained in specialized plasma membrane microdomains called caveolae. They are known to be expressed in endothelial cells and adipocytes and to play a role in endocytosis and oncogenesis (Cohen et al., 2003). In zebrafish, *caveolin-1* (*cav1α*) expression has been studied and its expression revealed to be absent from the migrating primodium but was detected in the mature neuromasts from 48 hpf. The expression pattern in the ALL and PLL neuromasts exhibits a ring-like shape, which would suggest that it is localized in the supporting cells (Nixon et al., 2007). The neuromast maturation was disrupted after knock down of *cav1α*. A decrease in the number of functional hair cells in the neuromasts of the PLL was observed at 72 hpf using a fluorescent dye. However, the migration of the primordium seemed not to be altered, as by 72 hpf, some neuromasts were observed in the tail but not along the body of the fish. This study showed a vital role of *cav1α* in neuromast development (Nixon et al., 2007). The exact function of *cav1α* in neuromast development is, however, not completely clear. Further investigations are needed to determine its role in either maturation or apical surface endocytosis of zebrafish hair cells.

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2.4.2. ru920 mutants (-myo6b)

Myosin VI has been found to play a role in hair cell mechanotransduction (Kappler et al., 2004). The authors were using the *ru920* mutant that showed an inability to response to vibrational and touch stimuli. The mutants circled back and swam upside down. Further analysis showed that, although the LL hair cells in the mutants developed normally, they displayed diminished ability to internalize a specific dye (DiAsp), which enters through mechano-electrical transduction channels. This suggested that the electrical response failed in the hair cells of the mutants. The mutation was localized in the *myo6b* gene, which is one of the two zebrafish orthologs of the human gene *myosin VI*, and the phenotype was confirmed by *myo6b* knock down. The authors speculated that the lack of Myo6b might disrupt the removal of the membrane from the apical surface by clathrin-mediated endocytosis and thereby cause stereociliary fusion.

2.4.3. mariner, sputnik, orbiter, mercury, gemini, astronaut, cosmonaut and skylab mutants

All these auditory/vestibular mechanosensory mutants were identified in a large-scale mutagenesis screen. They display defects in balance and acoustic startle reflexes owing to peripheral defects in the auditory/vestibular system (Nicolson et al., 1998). When these mutants were tested for their ability to internalize a marker for endocytosis, FM1–43, the majority of them showed a defect in apical endocytosis.

The *mariner* and *sputnik* mutants have splayed hair cell bundles and reduced or absent extracellular receptor potentials. The *mariner* mutant showed to be defective in *myosin VIIA*, which is expressed in the sensory hair cells (Ernest et al., 2000). This mutation correlates with the *shaker-1* mice mutants, which are deaf. The defect of *sputnik* is characterized by missing the link between the hair cells extremities, called tip link (Sollner et al., 2004). As mentioned before, it is caused by mutations in the *cdh23* gene. The Cdh23 protein is concentrated near the tips of hair bundles. The absence of tip links in *sputnik* larvae showed that the mechanotransduction but not the hair bundle integrity was affected. Cdh23 is therefore an essential tip link component required for hair-cell mechanotransduction.

Three mutants, *orbiter*, *mercury* and *gemini*, have normal hair cell morphology, but reduced or absent extracellular receptor potentials. Mutants from the third group, *astronaut* and *cosmonaut*, also appear to have normal hair cell morphology, and also have conserved their ability to generate extracellular receptor potentials. Astronaut and cosmonaut mutants are, however, partially or completely vibration insensitive. Loss of behavioural responses in these mutants presumably involves defects in later aspects of signal transduction, such as synaptic transmission. A different defect, namely degeneration of sensory hair cells have been shown in the *skylab* mutant (Nicolson et al., 1998).

When the *mariner, sputnik, orbiter, mercury*, and *skylab* mutants were treated with streptomycin, their reduced endocytosis ability was revealed, as they were protected from the cytotoxic effect of this aminoglycoside antibiotic. The effects of similar ototoxic compounds are further discussed in the next section of this review. Other chemicals that could affect the apical surface endocytosis could be deciphered using these mutants.

As described above, the development of the LL organs occurs in subsequent steps in which various genes are involved. Because of this already substantially combined molecular-physiological insight in the normal developmental process of neuromasts, it is possible to perform mechanistic toxicological studies on the impact of pollutants on disturbed LL development. Different modes of toxic action that occur during the different developmental processes can be studied in greater detail. In the next section, the toxicological studies, that have been reported so far, are reviewed and classified by their biological effects on the neuromasts.

3. Neuromast disruption after exposure to toxicants

Toxicological studies nowadays face the challenge to assess subtle and chronic effects caused by a mixture of chemicals present at low concentrations. The subtle effects of environmental compounds on fish's nervous or sensory system and behaviour have been difficult to assess in the aquatic environment, mainly because the measurement of these endpoints are complex. For this reason, there is a need to develop tools for detecting sensory toxicity. The sensory LL system of fish could be one of such tools. Proper development and function of neurosensory systems such as the LL neuromasts is essential for the ability of organisms to cope with their environment. Therefore, the LL system may serve as experimental screen for chemicals potentially interfering with neurosensory/auditory development and/or function. The molecular mechanisms of the LL development have been fairly well studied with the use of diverse developmental mutants or morphants, as reviewed in Section 2. Therefore, these models can further be used to decipher molecular mode of actions of toxic compounds on the LL system.

In particular, the hair cells of zebrafish have been shown to be sensitive to the action of drugs and heavy metals. For instance, for aminoglycoside antibiotics, such as gentamicin, intensively used clinically because of its broad spectrum of antibacterial actions, it has been found that some patients suffer from severe hearing problems (Forge and Schacht, 2000). Experimental studies using zebrafish have demonstrated that aminoglycoside antibiotics affect the LL system (Owens et al., 2008). In addition, heavy metals were shown to affect the sensory system in humans and orientation and olfactory system in fish (Hansen et al., 1999; Gobba, 2003). Similar to the antibiotics, heavy metals affect the hair cell development of zebrafish. The following section discusses the reports classified by the type of effects that have been observed, i.e. hair cell loss, the potential of hair cell to regenerate and the effects on behaviour related to LL dysfunction after exposure to toxicants.

3.1. The effects of toxicants on hair cell disruption

The overall list of toxicants that are known so far to affect the hair cells in zebrafish is presented in Table 2 and described in the following paragraphs.

3.1.1. Pharmaceuticals

There is an emerging evidence that pharmaceutical compounds are relatively widespread in the surface waters of many countries, especially in water bodies receiving effluent from sewage treatment facilities (Ritter et al., 2002). Antibiotics are an important group of pharmaceuticals in today's medicine. In addition to the treatment of human infection, they are also used in veterinary medicine. The relevance of antibiotics in the aquatic environment can be observed by regarding the amounts that are used and their ecotoxicological effects (Hirsch et al., 1999).

3.1.1.1. Aminoglycoside antibiotics. This class of antibiotics is the best documented type of antibiotics known to cause hearing loss in vertebrates. An evaluation of the emission of neomycin and streptomycin was performed in a recent study, and it was shown that these aminoglycosides imposed both acute and chronic risks to the aquatic environment. The authors considered the presence of these chemicals to be of significant environmental concern (Turkdogan and Yetilmezsoy, 2009). Aminoglycosides are produced by different strains of soil actinomycetes. All natural and semi-synthetic aminoglycosides share a similar structure consisting of several, usually

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Table 2
Compilation of studies detecting neuromast disruption in 4–5 dpf zebrafish (*Danio rerio*) upon exposure to various compounds.

Compounds	Concentration	Time of exposure	Measured endpoint [a]	Detection method	References
Aminoglycoside antibiotics					
Neomycin	10 μM	24 h	E	DASPEI	Ton and Parng (2005)
	50, 100, 150, 200, 400 μM	1 h	D	DASPEI, TEM	Murakami et al. (2003)
	10, 50, 100, 125, 250, 300, 500 μM	1 h	D	DASPEI, SEM	Harris et al. (2003)
	10 μM	1, 5 h	C	BrdU, IHC anti-PH3, acridine orange	Williams and Holder (2000)
	100 μM	1 h	F	YO-PRO-1, FM1–43FX	Chiu et al. (2008)
	25, 50, 75, 100, 200, 400 μM	30 min	F	YO-PRO-1, DASPEI, FM1–43	Owens et al. (2008)
	25, 50, 100, 200, 400 μM	1 h	F	FM1–43, TO-PRO-3, YO-PRO-1	Santos et al. (2006)
Gentamicin	5 μM	24 h	E	DASPEI	Ton and Parng (2005)
Anti-cancer drugs					
Cisplatin	50 μM	24 h	E	DASPEI	Ton and Parng (2005)
	100 μM	1 h	F	YO-PRO-1, FM1–43FX	Chiu et al. (2008)
	250, 500, 750, 1000, 1500 μM	4 h	F	FM1–43FX	Ou et al. (2007)
	100, 200, 300, 400 μM	4 h	F	DASPEI	Owens et al. (2008)
Vinblastine sulfate	100 μM	24 h	E	DASPEI	Ton and Parng (2005)
Antiprotozoal					
Quinine	200 μM	24 h	E	DASPEI	Ton and Parng (2005)
Estrogen					
Estradiol valerate	100 μM	1 h	F	YO-PRO-1, FM1–43FX	Chiu et al. (2008)
Heavy metals					
Cadmium	0.2, 5, 125 μM	3 h	I	ISH *hsp70*, *hsp70*/eGFP	Blechinger et al. (2007)
	5 μM	120 h	I	ISH *mt*	Chen et al. (2007)
Copper	1, 10, 50, 250 μM	2 h	B	DiAsp, acridine orange	Hernandez et al. (2006a)
	1, 10 μM	2 h	F	ET4, ET20 transgenic line, FM1–43, ISH *atoh1a, eya1, cldb*, IHC anti-Cldb	Hernandez et al. (2006b)
	68, 244 μM	120 h	A	DASPEI	Johnson et al. (2007)
	5–65 μM	5 h	H	DASPEI, SEM	Linbo et al. (2006)
Zinc	50, 250 μM	2 h	B	DiAsp, acridine orange	Hernandez et al. (2006a)
	100 μM	120 h	I	ISH *mt*	Chen et al. (2007)
Iron	50 μM	2 h	B	DiAsp	Hernandez et al. (2006a)
Silver	1, 50, 250 μM	2 h	B	DiAsp	Hernandez et al. (2006a)

[a] Measured endpoints the numbers correspond to: (A) % of visible neuromast. (B) Number of ALL and PLL neuromast. (C) Number of central cells and peripheral cells. (D) % of normal or reduced stained neuromast. (E) Average of fluorescent intensity in 10 larvae. (F) % of survival hair cells in 4 neuromast except for Hernandez et al. (2006b), where two neuromasts were measured. (G) Mean number of hair cells. (H) Mean number of hair cell per neuromast. (I) Imaging of reduced fluorescent dye.

three, rings. The hallmark of aminoglycosides, which chemically might better be termed aminocyclitols, is the presence of amino groups attached to the various rings of the structure. These amino groups and the additional hydroxyl groups convey the major chemical properties, namely high water solubility and a basic character (Forge and Schacht, 2000). The structural features determining ototoxicity remain unknown.

A number of studies showed that aminoglycosides that affect zebrafish hair cells, also affect human inner ear (Harris et al., 2003; Murakami et al., 2003; Ton and Parng, 2005; Chiu et al., 2008; Owens et al., 2008). In all of these investigations, dose-dependent effects on hair cell loss in fish treated with neomycin were observed. Also gentamicin and streptomycin induced hair cell loss (Ton and Parng, 2005; Seiler and Nicolson, 1999). These studies usually assessed the hair cells with fluorescent live staining and in some cases the absence of hair cells were confirmed by transmission electron microscopy (TEM) and SEM (Murakami et al., 2003; Harris et al., 2003). Similarity of the action of these compounds in fish with the ototoxic effects reported in humans suggests that zebrafish LL may be a suitable screen not only for fish but also for other vertebrates.

There are some reports showing developmental differences in sensitivity to aminoglycoside induced hair cell death. In mammals and birds the auditory hair cells become susceptible to the drugs as soon as they begin to function as mechanosensory receptors (Friedmann and Bird, 1961; Raphael et al., 1983). Similarly, there is a period of insensitivity to aminoglycosides in zebrafish LL. It is hypothesized that hair cells are at several stages of development within any given neuromast at the time of treatment (Harris et al., 2003; Murakami et al., 2003). It has been analysed whether there is a link between developmental insensitivity to aminoglycosides and mechanotransduction-dependent activity of hair cells (Santos et al., 2006). There were no age-dependent differences in the uptake of a mechanotransduction indicator, the fluorescent dye FM1–43, during the time when stage-dependent differences in susceptibility to neomycin were observed. This phenomenon is also not due to differences in overall maturation of the larvae. Rather, it has been shown that the difference in maturation of individual hair cells, independent of mechanotransduction activity, is determinant. Further research is needed to completely elucidate the basis of immature hair cell resistance to aminoglycoside treatment, for which the specific cell markers presented in the previous section could be used.

3.1.1.2. Other pharmaceuticals affecting the hair cells. Besides aminoglycosides, diverse other medicines have been shown to cause ototoxicity in humans, including the antimalaria drug, quinine, or the well-known aspirin. For a complete list, see the review of Forge and Schacht (2000). Interestingly, cisplatin and vinblastin sulfate, anti-cancer drugs, as well as quinine, were also shown to cause hair cell loss in zebrafish LL neuromasts (Ou et al., 2007; Ton and Parng, 2005; Owens et al., 2008). The authors showed that cisplatin-induced hair cell death occurs in a dose-dependent fashion in the zebrafish LL, in a way comparable to that in mammals.

In a recent study, potential neurotoxic chemicals were screened, and a range of chemicals affecting hair cells has been discovered in zebrafish (Chiu et al., 2008). The authors screened for hair cell toxicity in the LL of zebrafish embryos at 5 dpf, using the library of 1040 Food and Drug Association (FDA)-approved drugs and bioactive compounds (Chiu et al., 2008). They discovered some already

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known ototoxic drugs to have a significant dose-response effect on the hair cells of the LL in zebrafish. Some compounds with unknown ototoxic effects in humans, including pentamidine, spermadine and vincamin, showed to affect the LL hair cells of zebrafish. Pentamidine and propantheline were further analysed in mammalian cell culture. Mature mouse utricle explants were exposed to these two potential candidate ototoxins, which indeed showed to negatively affect mammalian hair cells. However, no significant hair cell loss was detected after treatment with cisplatin, which has been previously shown to cause hair cell loss in zebrafish (Ou et al., 2007).

Surprisingly, estrogen (estradiol valerate) has been identified in this study as also being potentially ototoxic with respect to hair cells. This contradicts with previous studies where estrogens were shown to have favourable vascular effects protective of hearing (Kilicdag et al., 2004; Hultcrantz et al., 2006). In addition, the ER β_2 knock down caused hair cell loss (see Section 2.3). These findings need further investigation, as it is most probable that hormone action depends on concentration and therefore contradictory effects may be produced. The knock down of other ER subtypes could be of great interest to investigate the impact of natural and synthetic estrogens on hair cell development in fish.

Screening for hair cell toxicity in the LL of zebrafish could help to decipher the adverse effects of many drugs or environmental compounds. Currently, no test for ototoxicity is included in drug evaluation protocols. Consequently, new drugs are completing clinical trials and reaching the public with no knowledge on ototoxic potential. Moreover, there is a growing environmental concern, as drugs can accumulate in the aquatic environment. Zebrafish could, therefore, be a model organism for enhancing drug tests or to assess the toxicity of the pharmaceuticals and their metabolites already present in the environment. Using the different mutants and morphants available to trace specific developmental processes, a more detailed analysis of the chemical impacts would be possible.

3.1.2. Heavy metals

Among toxic metals, cadmium has been associated with olfactory and sensory dysfunctions in humans as well as in zebrafish (Gobba, 2003; Blechinger et al., 2007). Using transgenic zebrafish strain that expresses the enhanced GFP (eGFP) under control of the *hsp70* gene promoter (hsp70/eGFP), the specific cell types that were affected after cadmium exposure could be identified, as the *hsp70* gene is known to be up-regulated in cells exposed to toxic metals. The cells that were centrally located in the neuromasts and possessed the typical shape of the mechanosensory hair cells, expressed higher levels of *hsp70* mRNA and consequently the hsp70/eGFP reporter gene. The sensory cells of the LL showed to be more sensitive to cadmium than the olfactory system. The authors suggested that cadmium might accumulate in the hair cells, as they showed *hsp70* accumulation and are in direct contact with the chemicals in the water (Blechinger et al., 2007). This study provides us with a rapid marker of cadmium exposure for early detection of sensory cells disruption in LL at concentrations below those that cause rapid and widespread cell death. This feature, allowing to test toxicity below the acute concentration, makes zebrafish LL very attractive to risk assessment.

Other metals also showed to affect the LL organs. A disruption in the neuromasts of zebrafish has been revealed after zinc exposure using a metallothionein (*mt*) ISH probe (Chen et al., 2007). Copper has been shown to affect mainly the olfactory system in the aquatic life. Studies using zebrafish showed that copper also affects the mechanosensory system (Hernandez et al., 2006a; Linbo et al., 2006; Johnson et al., 2007). Even if copper exposure at low concentrations produced no significant effects on growth, morphology or survival, it reduced the number of functional neuromasts. This had the implication that the fish exposed to copper had a reduced ability to orientate in current, which seriously compromised survival. Also iron and silver revealed to affect hair cell development in fish (Hernandez et al., 2006a; Chen et al., 2007). Altogether, these studies indicate that the heavy metals can cause profound effects on mechanosensory cells of the zebrafish LL, further supporting the suitability of neuromasts assessment for evaluating aquatic pollutants.

3.1.3. Mechanisms of hair cell disruption

Mechanisms of hair cells disruption appear to involve the formation of free radicals and the apoptotic cell death pathways (Forge and Schacht, 2000). It seems that the hair cell death is a consequence of the free oxygen radical formation known to create a chain reaction and cause oxidative stress. The typical morphological features of apoptosis have been observed in hair cells exposed to aminoglycosides (Forge, 1985). The aminoglycosides form a complex with iron and activate molecular oxygen, which in turn is reduced to superoxide by an electron donor. One consequence of free oxygen radicals production in the cells is the calcium influx disruption, which may result in blocking of cation channels located at the apices of the stereocilia of neuromasts hair cells in the fish LL system (Hudspeth, 1989; Kroese et al., 1989; Forge and Schacht, 2000). This biochemical alteration leads to hair cell loss in zebrafish exposed to ototoxicants. Free-oxygen-radical production is, therefore, the most probable reason of ototoxicity. This mechanism is supported by the finding that transgenic mice overexpressing the antioxidant enzyme superoxide dismutase are protected from aminoglycoside-induced hearing loss (Sha and Schacht, 1997).

The mechanism by which metals could affect the LL organs is also not completely elucidated. One theory is that cadmium and copper, as well as cobalt and mercury, interfere with the calcium uptake in the hair cells, as it has been shown with the aminoglycosides (Karlsen and Sand, 1987; Liang et al., 2003; Griesinger et al., 2002). The metal ion blocks calcium channels located on the surface of the hair cells by competing with calcium cations at stereocilia level. This disrupts the ion flux and causes hair cell dysfunction (Karlsen and Sand, 1987). Cadmium has been already shown to be a calcium antagonist at the level of the gills (Verbost et al., 1987, 1988). So, as the calcium ions play a preponderant role in signal transduction mechanisms in neuromast hair cells in the fish LL system (Hudspeth and Corey, 1977; Jorgensen, 1984), cadmium ions might affect mechanoreception and thereby alter the behaviour of fish exposed to them.

Even if the mechanism of toxicity of aminoglycosides and heavy metals is proposed, the number of tools available for studies on zebrafish neuromasts toxicity could help to decipher further mechanisms by which toxicants could affect the neurosensory system of fish.

3.2. Hair cell regeneration after toxicant exposure

Some of the experiments described above analysed also the hair cell recovery. The first study revealed that there are two kinds of cell populations, one dying, and the other proliferating (Williams and Holder, 2000). The authors suggested that the dying population of cells are hair cells and the proliferating cells are the periphery cells, called supporting cells. The supporting cells are thought to give rise to hair cells following hair cell death, creating a constant turnover in neuromasts, as already shown in Balak et al. (1990). The recovery of damaged LL was further analysed in the following studies. Zebrafish larvae were checked for the number of hair cells at 4 and 12 h after treatment with low concentrations of neomycin. It was found that after 12 h of recovery more hair cells were stained (Murakami et al., 2003). The authors explanation was that supporting cells are continuously adding new hair cells to their neuromasts. Since immature hair cells may not be as susceptible to aminoglycoside-induced death, the cells displaying DASPEI labelling at 12 h that

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Table 3
Effects of metals on mechanosensory behaviour in several teleost species.

Compounds	Concentration	Time of exposure	Age and species	Description of disruption	Detection method	References
Cadmium	5 μg/L	4 h	Adult sea bass *Dicentrarchus labrax*	Altered escape behaviour (neuromast tissue damage)	Escape behaviour in response to a water jet	Faucher et al. (2006)
	2 μg/L	72 h	Juvenile banded kokopu *Galaxias fasciatus*	Alteration of rheotactic behaviour	Rheotaxis observation	Baker and Montgomery (2001)
Copper	68, 244 μg/L	120 h	120 hpf zebrafish *Danio rerio*	Disruption in rheotaxis (loss of neuromasts)	Observation in water current	Johnson et al. (2007)

were not labelled at 4 h may be those that differentiated over this period (Murakami et al., 2003). Harris et al. (2003) also proposed that the youngest hair cells within a given neuromast might survive drug treatment. After neomycin treatment and after 24–48 h of recovery, hair cells also regenerated in this study (Harris et al., 2003).

Hernandez et al. (2006) identified the doses and times of copper exposure that cause reversible and irreversible damage to the zebrafish LL hair cells. As already shown with ototoxic drugs, zebrafish sensory hair cells are able to regenerate. The toxicants, beside inducing hair cell death, also stimulate proliferation among supporting cells in neuromasts, which then over time leads to the generation of new ciliated receptor cells (Williams and Holder, 2000). Hernandez et al. (2006a,b) observed that a regeneration potential was dose-dependent and was different between the ALL and the PLL. Authors suggested that high doses of the metal are able to destroy both the hair cells and most of the cells that have the capacity to generate new hair cells in the PLL. In contrast, in the ALL, even after high doses, abundant numbers of proliferating cells were detected. One explanation could be that ALL neuromasts are embedded in pits or canals in the head (Webb and Shirey, 2003), possibly offering more protection from external toxicants (Hernandez et al., 2006a). In further investigation, transgenic fish that express GFP in different cell types in the LL system were treated with copper (Hernandez et al., 2006b). ET4 transgenic fish have fluorescent hair cells, and ET20 are positive in their supporting cells. After exposure to copper, the hair cells, but not the supporting cells, that were able to give rise to new hair cells, showed to be affected.

The expression of proneural genes, like *atoh1a* and *eya1*, required for cell survival, was also lost after copper treatment. Both genes reappeared during recovery, first *eya1* and then *a* toh1a. This study also showed that *sox2* and Sox protein are expressed in the supporting cells. This was shown using the ET4 and ET20 transgenic lines, where Sox2 expression was not colocalized with the fluorescence of ET4 hair cells, but partially colocalized with the fluorescent supporting cells in the ET20 (Hernandez et al., 2006b). The authors hypothesized that the supporting cells expressing Sox2 could give rise to new hair cells after damage and support the regeneration in zebrafish neuromasts. This assumption was verified by detecting Sox2 expression after copper treatment. At all concentrations, Sox2 expression was detected, which supports the idea that supporting cells survive the treatment and then serve as hair cell precursors.

A very recent study showed that hair cells can regenerate after neomycin treatment from a transient increase in supporting cell proliferation which is accompanied with an up-regulation of *n* otch3, *deltaA* and *atoh1a* (Ma et al., 2008). It could be shown that Notch signalling limits the number of hair cells produced during regeneration by regulating supporting cell proliferation.

3.3. Effects of toxicants on mechanosensory behaviour

Changed behaviour is a sensitive target of environmental pollutants. Further, it may serve as a link between physiological and ecological processes (reviewed by Scott and Sloman, 2004). Behavioural endpoints may indicate subacute effects of chemicals, like the effects of toxicants on the sensory system. In order to know, whether the cellular effects on the LL have a toxicological relevance, the studies dealing with behavioural changes related to a LL dysfunction are presented in this section and in Table 3.

In the sea bass (*Dicentrarchus labrax*) the exposure to high concentration of cadmium resulted in severe neuromast tissue damage and an altered escape behaviour in response to a water jet stimulus (Faucher et al., 2006). Also in a study with the banded kokopu (*Galaxias fasciatus*), cadmium exposed fish showed an alteration of rheotactic behaviour (ability to orientate in current), which was attributed to a disruption in the LL (Baker and Montgomery, 2001). In zebrafish, a link between loss of neuromast function and rheotaxis disruption has been established after copper treatment (Johnson et al., 2007); the time spent at equilibrium was reduced in zebrafish embryos exposed to copper.

These studies showed that besides observing toxic effects on the molecular and cellular levels, the LL system dysfunction has functional consequences in exposed organisms. It is therefore important to assess hair cell/neuromast dysfunction to detect subtle effects of chemicals.

3.4. Further remarks

The range of chemicals that affect the LL organs might be broader than discovered so far. The toxicants that can affect the structure or function of the CNS and/or PNS, the so-called neurotoxicants (Tilson and Cabe, 1978; Tilson, 1993; Spencer et al., 1986), could as well affect the LL system of fish in general. In fact, exposure to neurotoxicants in humans can result in sensory, motor and cognitive dysfunction. Neurotoxicants like lead, methyl mercury, polychlorinated bisphenyls (PCBs) and environmental tobacco smoke affect the hearing system in humans (Lanphear et al., 2005). PCBs have also shown to accumulate in neuromasts of damselfish embryos living in PCB contaminated sites (Lobel and Davis, 2002). Recent investigations using crude oil exposure revealed effects on the sensory LL system in zebrafish as well (unpublished data).

Very little is known on how aquatic toxicants impact the sensory system in aquatic organisms. The use of zebrafish neuromast as biological endpoint is not yet a standardized test in ecotoxicology. But as mentioned already, many exposure studies have shown the suitability of this system for assessing potential sensory- or neurotoxicants. In fact, chemicals are in direct contact with these superficial organs, which showed to be very sensitive. Dysfunction in sensory and/or nervous system can have dramatic impacts on survival of fish species since many different vitally important behaviours such as feeding, reproduction, predator avoidance and rheotaxis rely heavily on olfactory and sensory cues (Scott and Sloman, 2004; Blechinger et al., 2007; Whitlock, 2006). It is therefore crucial to study toxic effects on neuromasts in order to best evaluate the impact of chemicals in the aquatic environment. The findings gained with the zebrafish as model can be extrapolated to other fish species, as the basic architecture and function of the LL is highly conserved across teleosts (Webb, 1989).

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4. In conclusion: linking developmental and toxicological studies

Having reviewed the abundant studies focusing on the neuromasts developmental process and the few investigations performed on neuromast disruption by toxicants, we propose the integration of both research fields. Potential links are explored below.

So far the toxicological analysis using neuromasts as endpoint assessed mainly the impact on hair cells differentiation. Two studies, intending to understand the process of hair cell regeneration, exploited the combination of chemical exposure with the molecular knowledge available. The first study analysed the impact of metals (Hernandez et al., 2006b). The expression of *atoh1a* and *eya1*, required for hair cell survival, was lost after copper treatment. The expression of *sox2*, a marker of supporting cells, was maintained. Indeed, the supporting cells were not affected by the treatment and could eventually differentiate into new hair cells after damage. Similarly, in a second study, an up-regulation of *atoh1a* and *notch3* after neomycin treatment was detected, accompanied with an increase in supporting cells proliferation (Ma et al., 2008). The treatment, therefore, activated a cascade of effects that finally caused hair cell loss followed by hair cell regeneration. Hereby, the link between both research fields provided an excellent base for understanding chemicals impact on a cellular level.

Moreover, the unexploited possibilities to assess chemical impact in detail are tremendous, as not only the hair cell differentiation, but also the overall process of neuromasts development can be investigated. The effects of toxicants on the ability of hair cells to perform apical endocytosis could be assessed looking at the different genes essential for this process, such as the myosins, caveolins or cadherins. Similarly, the impacts of pollutants on the placode formation or general neurogenesis could be measured using the proneural genes, such as *ngn1* and *nrd*. The damage caused to the primordium migration and cell deposition could be carefully assessed with *sdf1, cxcr4* and *cxcr7* gene expression.

The subtle effects of pharmaceuticals, industrial and agricultural chemicals and their by-products are only starting to be realised. Regulatory guidelines for aquatic pollutants are usually based on apical endpoints such as acute lethality, ignoring the effects appearing at much lower concentrations, causing "ecological death", as discussed in the review of Scott and Sloman (2004). The objective of their review was to point at the behaviour as an integrative tool, allowing to test the subacute effects of pollutants. Similarly, the process of neuromasts development enables to test the effects of chemicals on behaviour, on hair cell differentiation, and on the other specific developmental processes in the LL system, not investigated so far. This approach gives the opportunity to assess biological endpoints that occur at low, and therefore, more environmentally relevant, concentrations. Most importantly, the increasing range of molecular markers and genetic tools available in zebrafish enables to identify specific mode of action of potential toxic compound. So far, only a small part of the molecular and genetic potential of mechanosensory mutants and morphants has been exploited for toxicological research. There are multiple unexplored strategies available that integrate the basic knowledge on neuromast development with chemical exposure.

The potential of mechanosensory hair cell disruption to assess neurosensory impact of toxicants has only marginally been exploited to date. More effort should be invested in this direction. Deciphering sensory dysfunction caused by chemical exposure would require increasing the knowledge on the modes of action of these toxicants by further studying the normal mechanism of neuromast development. An integration of developmental and toxicological studies will provide a powerful tool for future risk assessment. The overall list of possibilities is far from being complete.

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