

Hair cell regeneration: winging our way towards a sound future

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The discovery of hair cell regeneration in the inner ear of birds provides new optimism that there may be a treatment for hearing and balance disorders. In this review we describe the process of hair cell regeneration in birds; including restoration of function, recovery of perception and what is currently known about molecular events, such as growth factors and signalling systems. We examine some of the key recent findings in both birds and mammals.

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Abbreviations

FGFR3 fibroblast growth factor receptor 3
Hes hairy and enhancer of split
MAPK mitogen-activated protein kinase
Math1 mammalian atonal homolog 1
TGF α transforming growth factor α
VOR vestibulo-ocular reflex

Introduction

The discovery of hair cell regeneration in the inner ear of birds following exposure to ototoxic drugs or intense acoustic stimulation forever changed our views on how hearing and balance disorders may someday be treated (Figure 1). The majority of disorders causing permanent hearing impairment and many balance disorders are thought to be due to degeneration of the hair cells — the mechanosensory receptor cells — of the inner ear sensory epithelia. In mammals, when these cells are lost due to genetic mutation, disease, exposure to environmental toxins or aging, hearing and/or vestibular impairments are permanent. In 1987–88, five seminal papers [1–5] on hair cell regeneration were published, establishing: first, that experimental destruction of hair cells in the mature avian cochlea (basilar papilla) stimulates the proliferation of support cells and new hair cell production; second, in the undamaged avian cochlea, there is virtually no mitotic activity; third, in the mature avian vestibular epithelium,

there is a low rate of cell cycle activity and ongoing production of new hair cells. These papers stimulated a new wave of research, the goals of which were, and still are: first, to understand hair cell regeneration in the inner ear of birds and other non-mammalian vertebrates; second, to examine the functional capabilities of the inner ear and neural pathways following regeneration; third, to stimulate replacement of lost or injured hair cells in the inner ear of mammals. In this review, we comment on recent progress in each of these areas.

Restoration of auditory and vestibular function

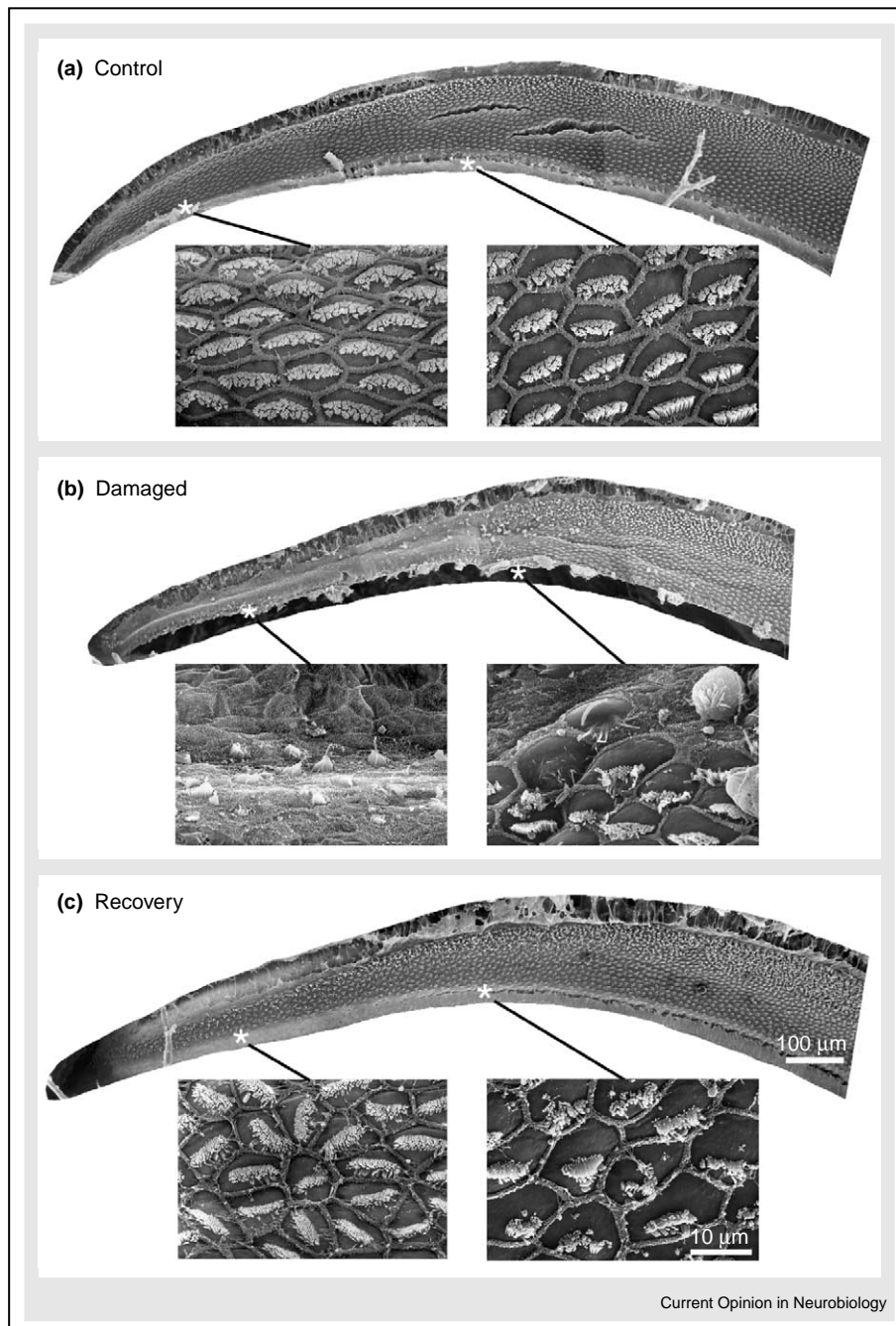
Once regeneration of hair cells in the inner ear of birds was confirmed, the question that immediately arose was: do the regenerated cells restore hearing and balance? To do this, new hair cells must develop appropriate ion channels for transduction and be re-innervated by the VIIIth nerve fibers that make appropriate connections in the CNS. Additionally, the animal must be able to access this information to make behaviorally meaningful responses. A thorough review of the literature up until 1999 on the recovery of auditory processing is available [6]. Table 1 categorizes this literature and provides additional references.

Recovery of sensory function: new cells or old cells with new life?

The earliest attempts to determine whether regenerated hair cells restore auditory function produced equivocal results. Studies from several laboratories showed recovery of electrical responses in the brain in response to acoustic stimulation and recovery of behavior following noise damage or aminoglycoside toxicity [7–10]. The problem was one of interpretation: is recovery of function mediated by the newly produced hair cells, or by recovery of hair cells and their associated structures that were injured, but not fatally so, by the intervention? When the time course of functional recovery precedes maturation of the new hair cells, interpretation leans toward recovery of damaged cells [11–14]. When functional recovery corresponds with the time course of new hair cell differentiation, or with the particular functional attributes of an area in which all the original hair cells were destroyed, interpretation favors regenerated hair cells as the transducers. It appears that both processes can mediate recovery under different circumstances.

Direct evidence for recovery mediated by the regenerated hair cells requires that all of the hair cells responsive to a particular sound attribute are destroyed, leading to a failure of the response (either physiological or

Figure 1



Scanning electron micrographs showing hair cell regeneration in the starling basilar papilla (BP) after aminoglycoside treatment. Each panel shows a low power scanning electron microscope image of the basal half of the BP, and two high power photomicrographs from the positions indicated. **(a)** shows the control untreated case; **(b)** shows a BP after drug treatment; and **(c)** shows a BP 20 weeks after drug treatment. Note that immediately after aminoglycoside treatment, almost all hair cells disappear from the basal end; apically, there is also a significant loss of hair cells. After 142 days **(c)**, a normal complement of hair cells returns, but the mosaic is somewhat abnormal. The asterisks indicate the approximate location along the BP of the high power images shown below. These images came from the work of Marean *et al.* [18**].

behavioral), and that this is followed by recovery of the response with a time course consistent with the production and differentiation of new hair cells. This scenario was partially achieved by some early studies using record-

ings of responses to pure tone stimuli [9,15], and has been more definitively shown by several studies examining the responses of single VIIIth nerve axons [6,16,17**]. Formally, however, one could still argue that the response

Table 1

Studies evaluating the functional properties of regenerated hair cells

Level of analysis	Dependent variable	Type of damage	Recovery period studied	References
Inner ear	Ototacoustic emissions	Intense sound exposure	1 wk - 8 wks	[52–54]
	Endocochlear potential	Aminoglycosides	1 wk - 22 wks+	[55–57]
		Intense sound exposure	0 day - 12 day	
Eighth nerve	Vestibular hair cell physiology	Aminoglycosides		[58,59]
	Compound evoked responses	Intense sound exposure	0 days - 30 days	[7,60–68]
		Aminoglycosides	2 days - 20 weeks	[9,10,69–72]
	Single unit responses	Intense sound exposure	0 days - 4 mo	[66,73–77]
CNS	Single unit responses	Aminoglycosides	2 days - 20 wks	[6,14,16,17**,21]
		Intense sound exposure	0 days & 12 days	[11,12]
	Metabolic influences	Intense sound exposure	2 days - 43 days	[78–81]
Behavioral studies	Basic psychoacoustics	Intense sound exposure	1 day - 4 mo	[8,18**,23,24,26,82,83]
		Aminoglycosides		
	Complex behaviors	Aminoglycosides	5 days - 23 wks	[25**,84]
	Vestibular reflexes	Aminoglycosides		[20,29,85]

properties of the cochlea change following damage to a subset of hair cells, allowing a different subset to restore the response properties initially lost. Such changes are not unprecedented. For example, the frequency/place representation in the cochlea of birds and mammals changes dramatically during the early stages of hearing development, and small reversible changes in the tonotopic representation in the brainstem auditory nuclei of chicks are reported after hair cell damage [12].

One way to conduct a definitive test of the involvement of regenerated hair cells in recovery of function is to remove the regenerated cells after restoration of function and observe the specificity of the changes. This was achieved by behavioral studies examining the loss and recovery of high frequency sensitivity in starlings after aminoglycoside treatment. After recovery stabilized, a second aminoglycoside treatment, designed to kill hair cells only in the area of regeneration, reversed the recovery of high frequency sensitivity [18**]. This result argues strongly for the involvement of the regenerated hair cells in recovery of auditory function in birds. Although evidence for the participation of regenerated hair cells in functional recovery is now convincing, the recovery is not perfect. Most studies have reported residual long-term deficits, such as mild sensitivity impairments and mild to moderate tuning impairments (e.g. see [17**]).

In summary, both behavioral and physiological studies provide convincing evidence that regeneration of hair cells restores near normal vestibular reflexes and response properties in the vestibular afferents to the brain [19–21]. Does this same process occur in mammals to a limited extent, and has gone unnoticed? Alternatively, is the process started and then aborted before the differentiation of new hair cells? One report [22] suggests that extensive regeneration of mammalian vestibular hair cells

can be induced by local application of growth factors and leads to recovery of vestibular reflexes. However, the lack of direct evidence for mitotic regeneration casts doubts on these claims.

Recovery of perceptual processing and behavioral plasticity

Although many reports have examined the role of hair cell regeneration in the recovery of sensory information processing, few have examined more complex properties of perceptual processing and behavioral plasticity. In general, studies have found that temporal and frequency resolution gradually return to normal or near normal in conjunction with the return of sensitivity [23,24,25**,26]. But what about the recognition and production of vocal signals that depend on hearing and are necessary for communication? And what about the stability of behavior, or its plasticity, as the receptor system and central pathways are experiencing such profound alterations in information flow? A few recent studies have examined these questions and provided new insights into, as well as new tools to investigate, old questions.

Dooling *et al.* [25**] used budgerigars (domesticated parakeets) to examine the perception of vocalizations. These birds are classical mimics who readily learn new vocalizations throughout life. Birds were trained to match their vocalizations to specific acoustic templates, and then subjected to a profound high frequency hearing loss by aminoglycoside injections. As their hearing recovered, the birds were retested with the same acoustic templates. The vocal mimicry initially declined, but was restored to pre-injection levels quite rapidly, before the full recovery of thresholds or discrimination abilities. This result suggests that relatively little acoustic feedback is necessary to guide previously learned vocal abilities, and that regeneration of only a few hair cells is sufficient to restore that feedback.

A second study examining complex communication behavior following hair cell regeneration was recently completed in our laboratory [27**]. Male Bengalese finches (*Lonchura striata domestica*) are songbirds that learn a single sequence of 'syllables' early in life from their father and reliably produce the same song throughout life, provided that hearing is normal. After recording each animal's song and verifying its stability, the birds were treated with a combination of low frequency noise exposure and aminoglycosides to cause a severe hearing loss that included both high and low frequencies. The songs rapidly deteriorated after the treatments, just as they do following surgical deafening [28]. As hearing was restored by hair cell regeneration, the song returned to its pre-exposure structure. Thus, restoration of hearing allows each bird to access a stored 'template' of its own learned vocalization and gradually match its new vocalizations to this stored memory. Remarkably, this period of hearing recovery also appeared to reinstate or unmask a capacity for behavioral plasticity not usually apparent in adult songbirds of this type. After the recovery of their normal (pre-deafening) song, some birds altered individual syllables so as to 'copy' portions of their cagemate's song. That is, as adults, they incorporated new elements from their acoustic environment into their otherwise stable song.

Hair cell regeneration in the avian vestibular system can also be used to study behavioral plasticity [29]. The vestibulo-ocular reflex (VOR) and the vestibulo-colic reflex normally disappear after hair cells in the crista of the semicircular canals are killed; these reflexes reappear as the hair cells regenerate (These reflexes involving the vestibular organs in the inner ear and the extraocular eye muscles (VOR) or the neck musculature (VCR) are essential for maintaining a steady gaze while the body is in motion) [19,20]. However, when an animal is sub-

jected to an environment devoid of smoothly changing visual stimuli, by maintaining it in stroboscopic illumination, normal VOR recovery fails to occur. Apparently, by depriving the animal of retinal slip information, the central gain control system cannot be calibrated. Remarkably, however, just 48 h (and maybe even less) of normal visual environment is long enough to reinstate a normal VOR. This study and the one by Woolley and Rubel [27**] discussed above suggest that hair cell regeneration in birds may be a very useful new way to study how sensory information shapes neural structure and function.

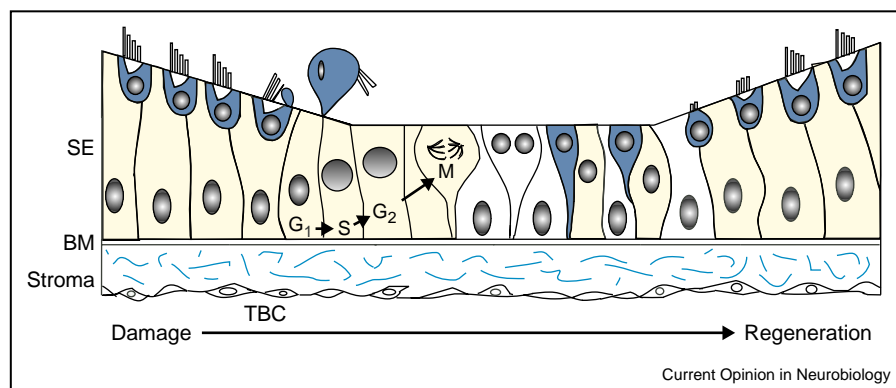
Molecular events leading to hair cell regeneration

The events leading up to the regeneration of the auditory and vestibular epithelia after damage in chicks have been the focus of much study; hopefully, understanding these phenomena will point the way to regeneration in the mammalian inner ear. To understand the molecular mechanisms of hair cell regeneration, three general approaches have been taken: the first looks at which proteins might be expressed or repressed during the regeneration process; the second examines the effects of exogenous signals such as growth factors on the regeneration process; and the third studies the intracellular signals that play a role in the progression of quiescent support cells into the G₁ and S phases of the cell cycle. Figure 2 provides a schematic of the steps involved in the regeneration of hair cells.

Proteins expressed during hair cell regeneration

Following the first approach, Lomax *et al.* [30] recently used differential display of expressed genes to show that a novel member of the ubiquitin ligase gene family is upregulated in response to noise-induced damage in the chick basilar papilla. In a directed screen for receptor

Figure 2



Schematic representation of hair cell regeneration after damage. Blue cells represent hair cells and yellow cells denote supporting cells. After damage, the hair cells are extruded to the lumen and some of the support cells are triggered to divide. The M phase of the cell cycle takes place at the luminal surface of the sensory epithelium. After division, the new cells, shown in white, go on to differentiate into hair cells and supporting cells. SE, sensory epithelium composed of hair cells and support cells; BM, basement membrane; TBC, tympanic border cells. Drawing courtesy of Dr Jennifer S Stone.

tyrosine kinase genes expressed in support cells, we [31] found that the growth factor receptor fibroblast growth factor receptor 3 (FGFR3), which is highly expressed in the support cells of the auditory sensory epithelium, is rapidly downregulated after damage, and begins to be expressed again after the cells exit the cell cycle. These results suggest that FGFR3 plays a role in maintaining support cells in their quiescent state. Interestingly, this same gene is found to be upregulated after noise damage in the rat; thus, a system that does not regenerate appears to regulate this gene in the opposite direction [32]. An actin-interacting protein, WD40 repeat protein (WDR1), is also upregulated in the supporting cells of the chick basilar papilla after noise damage [33]. The expression of this gene is likely involved in actin turnover; the investigators propose that it might be important in the restoration of cytoskeletal integrity after damage. Many other developmentally important genes are likely to be upregulated in the regenerating regions of the basilar papilla. For example, Delta1 and Notch1 expression are upregulated during the process of regeneration, when new hair cell genesis is at its peak [34].

The role of signaling molecules and growth factors in hair cell regeneration

To identify factors that might promote regeneration, several investigators have tested defined signaling molecules and growth factors in assays of hair cell regeneration. Insulin-like growth factor 1 stimulates DNA synthesis in the chick vestibular sensory epithelium in a dose-dependent manner [35]. This factor has also been shown by PCR analysis to be upregulated after damage [36]. By contrast, FGF2 inhibits DNA synthesis in avian vestibular and auditory sensory epithelia [37]. IGFs and FGFs are important regulators of progenitor cell mitotic activity in other regions of the nervous system; thus, these results again demonstrate that regeneration is likely to be regulated by the same factors normally involved in embryonic development.

It has been known for some time that transforming growth factor α (TGF α) and epidermal growth factor in the presence of insulin stimulate cell proliferation in the cultured mature mammal vestibular epithelium [38,39]. Recent studies show that infusion of TGF α and insulin directly into the inner ear of adult rats stimulated DNA synthesis in the vestibular sensory receptor epithelium [40]. Corwin and colleagues [41–43] investigated various intracellular signal transduction pathways using *in vitro* cultures of utricular sensory epithelial sheets derived from both mature avian and neonatal mammalian inner ears. Although cell proliferation in both the avian and mammalian sensory epithelia was reduced by inhibitors of several key signaling intermediates, including phosphatidylinositol 3' kinase (PI3K), target of rapamycin (TOR), mitogen-activated protein kinase (MAPK), and protein kinase C [41–43], these investigators found that the

MAPK pathway plays a more significant role in the avian cultures than in similar mammalian cultures.

Cell cycle regulation in hair cell regeneration

Once support cells in the sensory epithelia are enticed to enter and progress through the cell cycle, it is necessary for one or both daughter cells to receive the correct signals to differentiate into hair cells. Recently, mammalian atonal homolog 1 (Math1), a basic helix–loop–helix transcription factor, has been shown to be necessary for hair cell differentiation; mice deficient in this gene fail to develop hair cells in either the auditory or vestibular epithelia [44**]. Another class of related molecules, mammalian hairy and enhancer of split homologs (Hes1 and Hes5), act as negative regulators of hair cell differentiation, and deletion of Hes1 and Hes5 in mice leads to an overproduction of hair cells [45**]. The precise control of these activators and suppressors of hair cell fate leads to the patterned array of hair cells that is critical to the proper functioning of this system. The experimental manipulation of this system has led to a potential strategy for hair cell replacement. Results from two groups show that overexpression of Math1 in cultures of neonatal mouse inner ear leads to the production of extraneurary hair cells from the greater epithelia ridge in the case of the cochlea, and from the support cells in the case of utricles [46*,47]. This is an exciting finding because it suggests the potential of replacing lost hair cells using endogenous tissue. It is not clear, however, whether these new hair cells would make the correct functional connections with the spiral ganglion.

Another strategy for hair cell replacement in mammals has emerged from recent studies of cell cycle regulators in the inner ear [48,49]. The numbers and timing of hair cell and support cell production in the auditory and vestibular epithelium is highly regulated. For example, in mice deficient for the cell cycle inhibitor p27 (Kip1), hair cells are initially overproduced; later, a massive degeneration of hair cells occurs, particularly in the basal region of the organ of Corti, leading to hearing impairment. This study [49] does, however, demonstrate that production of hair cells can be extended into the postnatal period under the right conditions.

The finding that stem cells can be isolated from the CNS has led to widespread speculation that they will lead to cures for many neurodegenerative diseases. Limited success has been achieved to date, but research in this arena is still in its formative stages. Recently Ito *et al.* transplanted neural stem cells into the mammalian inner ear [50**]. They showed that these cells survive and appear to integrate into various structures up to four weeks after transplant. They even saw some cells that appeared to take on the morphology of hair cells. Although these findings are encouraging, the extent to which such cells can develop into specialized sensory cells is unclear. For

example, stem cells transplanted into the eye integrated and took on the morphology of retinal neurons but failed to express any retinal-specific markers [51].

Conclusion and future directions

In summary, over 15 years of studies on hair cell regeneration in the inner ear of birds has taught us that a quick and easy 'cure' for sensory neural hearing loss is unrealistic. It is impossible to predict when these efforts will lead to a therapy for the hearing impaired. However, 15 years is but a short time in the history of science and great progress has been achieved in these years. The phenomenology of hair cell loss and regeneration has been well described in birds; new *in vitro* and *in vivo* preparations have been developed and cell lines are beginning to become available. Functional recovery due to regenerated hair cells has been confirmed and we are beginning to unravel some of the molecular signals that stimulate and inhibit regeneration in birds. Limited postnatal proliferation and ectopic hair cell expression has been achieved in mammals. Of most importance, hair cell regeneration has become a legitimate and exciting field of interest and exploration. That is the major change in the field that will someday lead to therapeutic interventions for the hearing impaired. The discovery of the structure of DNA was only half a century ago, yet we seem on the verge of promised cures for various conditions. It is exciting to speculate on the impact the next half-century of research will have on hair cell regeneration.

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