Some of past achievements are summarized below: from the ear to the eye, and vice-versa

Our projects are aimed to understand how the auditory and visual sensory cells develop and function. Keen attention is paid to how the hearing and vision organs have changed and adapted during evolution. Notably, how the sound-receptive (the hair bundle) and the light-sensitive (the outer segment) structures (Fig. 1A,B) are tuned to the specific needs of corresponding sensory modalities, and how that might have impacted the disease-linked molecular networks?. Indeed, very often, evolutionary selection constraints and pressures proceed by "modifications" of themes that first arose in other systems and/or other species, making optimal use of natural structural variations, and/or reuse of existing molecules.

Over the last years, we contributed to the elucidation of the disease mechanisms of dozen deafness genes, several of which are involved in Usher syndrome (deafness and blindness in humans: see below). Resorting to many of the identified genes as entry points has enabled us to enlighten both fundamental and medical aspects of the auditory and visual systems.

**A giant spectrin defies convention in auditory and visual sensory cells**


The remarkable hearing capacities of mammals arise from various evolutionary innovations. These include the cochlear outer hair cells and their singular feature, somatic electromotility, i.e. the ability of their cylindrical cell body to shorten and elongate upon cell depolarization and hyperpolarization, respectively (Ashmore et al., 2010). This sound amplification process, driven by transmembrane protein prestin, is associated with the presence of a flexible cortical network of F-actin and spectrin along the OHC lateral wall. The precise composition of this spectrin-based submembrane cytoskeleton and how it is linked to the motor protein prestin have been unknown.

**Fig. 2:** Based on analogies with membrane deformations in red blood cells, it had been assumed that the OHC cortical lattice includes conventional spectrins, βII and αII subunits. Surprisingly though, we found that of the five known spectrin β subunits, only the unconventional spectrin βV that is twice the length of conventional β spectrins is concentrated at the OHC lateral cortical lattice. Spectrin βV did associate with prestin, which sheds light on the molecular bases of mammalian sound amplification (Legendre et al. 2008). In two parallel studies conducted with 2 PhD students (Papal S & Corteste M), we later showed that, unlike in auditory OHCs, spectrin βV is cytoplasmic in photoreceptor cells, forming a spectrin cargo adapter that bridges components of the phototransduction machinery to the actin- and microtubule-based motors. We proposed that this spectrin enables protein/organelles transport up to the photoreceptor outer disks, where phototransduction occurs (Papal et al., 2013). More recently, phylogenetic analyses of spectrin sequences revealed strong signatures of adaptive evolution at multiple sites along βV amino acid sequence in the lineage leading to mammals.
This was accompanied with substantial differences in the subcellular location of this protein between the frog and the mouse vestibular and cochlear hair cells (Cortese et al., 2017) (see side image). Our results support a scenario where the singular organization of the cortical cytoskeleton at the OHC lateral wall may have emerged from molecular networks initially involved in membrane trafficking, which were present near the apical junctional complex in all frog hair cells and would have subsequently expanded to the entire lateral wall in the mammalian auditory OHCs.

Control of stereocilia length and hair-bundle shape in the hearing organ by Class III myosins


Cellular actin-rich protrusions (e.g. brush border microvilli, growth cones, hair bundle) are critical to a wide range of biological functions, thanks to controlled variations in the dimensions, dynamics and positioning of these actin structures within cells. In the inner ear, the sound mechano-sensitive hair bundle, is made up of arrays of mechano-sensitive microvilli-like protrusions, stereocilia, exhibiting a precise graded spatial arrangement and size, which are vital for its role in converting mechanical stimuli like sound to neural signals. The importance of actin-based cytoskeleton for hearing is reflected in the increasing number of disabling mutations in several actin-binding proteins (espin, eps8, erzin, fascin, fimbrin) and the actin-based motor proteins (MyH9, MYH14, MYO3, MYO6, MYO7, or MYO15) causing deafness in human and mice (see http://hereditaryhearingloss.org/). Myosin IIa (defective in the late-onset deafness form DFNB30) and its paralog myosin IIIb are both expressed at the stereocilia tips of the developing hair bundle, but their precise role and underlying pathogenic mechanisms have remained elusive.

Together with A. Lelli (postdoctorant), we studied the hearing abilities of single (Myo3a<sup>−/−</sup>, and Myo3b<sup>−/−</sup>) and double knockout mice. We found that, unlike the situation in previous in cellulo studies on filopodia, the actin-crosslinking protein espin-1 is properly targeted to stereocilia tips in <i>vivo</i>, even in the absence of the two myosins.

**Fig. 3**: The Myo3a<sup>−/−</sup>Myo3b<sup>−/−</sup> mice were profoundly deaf. Albeit Myo3a<sup>−/−</sup>Myo3b<sup>−/−</sup> cochlear hair bundles display robust mechanoelectrical transduction currents with normal kinetics, their morphology is severely affected starting at embryonic stages, with abnormal structural features that are highly dynamic: these include abnormally tall and numerous stereocilia, ungraded stereocilia bundles (see side image), and bundle rounding and closure (instead of V-like shape). On the other hand, Myo3b<sup>−/−</sup>Myo3a-cKO mice that lack myosin IIb and lose myosin IIa postnatally exhibited normal hearing. We conclude that the two class III myosins are required for size and shape control of the hair bundle. Unexpectedly, instead of promoting growth, as previously thought, the two myosins act redundantly to limit the elongation of stereocilia and of subsequently regressing microvilli; this contribution, essential for bundle shaping, is necessary only at the very early stages of bundle morphogenesis (Lelli et al., 2016).

Hidden hearing impairment unraveled by studies of specific deaf mutant mice


Two PDZ-domain containing sub-membrane scaffold proteins defective in Usher syndrome (USH), harmonin (responsible for USH1C) and whirlin (USH2D) have been found to play a key role at distinct positions along the length of the stereocilia, controlling bundle shape and/or stereocilia dimensions (Boeda et al., 2002;
Mburu et al., 2003). We sought the existence of a “PDZ code” within the developing stereocilia contributing to the control of membrane to cytoskeleton cross-linking. Seeking new PDZ proteins of the auditory hair bundle, we identified (together with K. Kamiya, postdoctorant) Nherf1 and Nherf2 as key partners for the Usher proteins, cadherin-23 (USH1D) and protocadherin-15 (USH1F), the two Ca^{2+}-dependent cadherins that form the tip-link, gating the mecano-electrical transduction channels. We found that the absence of either Nherf1 or Nherf2 causes defects in polarity and symmetry of the hair bundles, respectively; pointing that Nherf1 and Nherf2 cannot substitute for one another in the developing cochlear hair bundles. Most importantly, while phenotyping the physiological features of Nherf1^{−/−} mice, we observed an intriguing discordance: the auditory brainstem response (ABR) in these mice revealed mild hearing impairment (loss of 20-25 dB for high frequency sounds), despite the fact that OHCs at the basal region of the hearing organ are totally non-functional (as revealed by the total loss of distortion product otoacoustic emissions (DPOAEs), Fig. 4). This configuration was reminiscent of frequent situations encountered during routine clinical and diagnosis audiology evaluations where patients with almost normal audiograms do experience, often for unexplained reasons, severe hearing problems in noisy environments.

![Figure 4: (A) ABR and DPOAEs thresholds in Nherf1^{−/−} mice. (B) Off-frequency hearing: the “still” healthy apical region of the cochlea compensates for the loss of activity at the cochlear base.](image)

We thus underwent more extensive studies on Nherf1^{−/−} mice aiming to uncover possible explanation for such unexplained clinical paradox. Of note, the hearing organ has a tonotopic map, with high sound frequencies processed at the base of the cochlea, and low frequencies at the apical region (Fig. 4B, left). Through a set of specialized physiological audiometric tests (compound action potentials (CAPs), CAP masking tuning curves; collaboration with P. Avan, Clermont-Ferrand), correlated with morpho-functional analyses we could show that the apical region of the cochlea in these mice was healthy, and that this apical region compensates the loss of activity at the base (Fig. 4B, right). This compensation fails to occur in the presence of interference from low-frequency sounds (i.e. such as noise), which would thus account for the difficulties some patients experience in noisy environments. We therefore proposed that patients with audiogram values discordant with hearing performance (vulnerable to interference by low-frequency, particularly in noisy environments) should be submitted to complementary clinical tests to evaluate their frequency selectivity, which might unveil potential off-frequency hearing.

Unraveling the origin of hearing and vision loss in Usher type I (USH1) patients

The Usher proteins as key players in the development and functioning of the hair bundle, the sound receptive microvillar structure

Congenital, profound hearing loss, balance deficiency, and retinitis pigmentosa starting before puberty and rapidly leading to blindness characterize USH1, the most severe of the three Usher clinical form (see Bonnet and El-Amraoui, 2012; El-Amraoui and Petit, 2014). At present, six USH1 genes have been identified; they encode the motor protein myosin VIIa, the scaffold proteins harmonin and SANS, cadherin-23 and protocadherin-15, and the calcium integrin binding protein CIB2 (http://hereditaryhearingloss.org/). Mutant mice defective for any of the first five known Usher 1 proteins — myosin VIIa, harmonin, cadherin-23, protocadherin-15, and SANS — all display profound congenital deafness, which we have ascribed to an early embryonic disorganization of the development and shaping of the hair bundle (see Fig. 5)(see Boeda et al., 2002), reviewed in Mathur, P., & J. Yang. 2015).
Fig. 5: In the model we proposed myosin VIIa is required to transport some Usher 1 proteins into the stereocilia. Cadherin-23 and protocadherin-15 form the early transient links connecting the apical regions of stereocilia. Myosin VIIa, harmonin-b (for which we demonstrated direct binding to F-actin), and sans anchor the cadherin-mediated links to the stereociliary actin filaments. The interstereocilium links are anchored to the cytoskeleton and help to maintain the cohesion of the growing and elongating stereocilia, to ensure the correct shaping of the hair bundle. The absence of any of the Usher1 proteins leads to the stereocilia growth disorganization, and hair bundle fragmentation thus resulting in total hearing loss.

Until recently, the origin of the pathological events leading to Usher 1 retinal dystrophy has remained elusive, probably because none of the Usher 1 mice display a visual defect. The question then is WHY?

Why do Usher 1 mutant mice have no visual defects? The calyceal process, a forgotten microvillus structure at the origin of Usher 1 retinopathy


Comparative analyses of the subcellular distribution of Usher 1 proteins in photoreceptor cells from various species singled out the presence/absence of a ring of microvilli structures, the calyceal processes, at the base of photoreceptor cells in primates and a possible cause for USH1 retinopathy (Sahly et al. 2012). Similar to the hair-bundle stereocilia, the calyceal processes, which were visible in macaque, but not mouse photoreceptor cells (Fig. 6A,B) are F-actin-rich membrane projections that emerge in the apical region of the inner segment, forming a collar ensheathing the base of the rod and cone outer segments. The absence of calyceal processes in mouse photoreceptors (Fig. 6B) probably accounts for the lack of retinal defects in mouse models of Usher 1 protein deficiency. This also pointed that despite processing different sensory signals — mechanical and photonic inputs —, the auditory hair cells and the retinal photoreceptors both harbor microvilli-cilium related structures interconnected by the Usher 1 protein network. A defect of Usher 1-mediated adhesion belt around the outer segment and the calyceal processes was proposed as a possible cause of the retinal dystrophy (Sahly et al., 2012).

Figure 6: The calyceal processes at the origin of Usher 1 retinopathy. (A,B) The F-actin labeled (red) calyceal processes (and their roots in the inner segment) are visible in monkey (A) but not mouse (B) photoreceptors. Usher 1 proteins, here USH1F, are detected in the calyceal processes, around the outer segment base.
As amphibians do have large photoreceptors with well-developed calyceal processes, we used a morpholino-based approach in *X. tropicalis* to knockdown Usher 1 gene expression. In the *pcdh15* morphant larvae, after knockdown of *pcdh15* expression, electroretinogram response measurements showed reduced amplitudes of the a-wave, indicative of dysfunctional photoreceptors. We showed that the calyceal processes were virtually absent in cone photoreceptors, and the F-actin cytoskeleton that forms the core of the calyceal processes and their roots was significantly decreased in both cone and rod photoreceptors. As for the impact on the outer segments: several geometric parameters were explored: in most morphant cones, instead of being straight, the outer segments were curved (Fig. 6A). Also, in many defective rods, the outer segment at the base displays oversized disks expanding beyond the edge of the inner segment. **We conclude that during the daily renewal of the outer segment, the Usher 1-mediated cohesion of the calyceal processes is necessary at the photoreceptor inner segment ridge, to control the size of the generated disks, and guide their upward movements into the outer segment to maintain proper size, and functioning.**

**References:**


