Surgical approach for a safe intra-cochlear injection of AAVs in *Macaca fascicularis*

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See presentation on Podium Session 13 - Emerging Gene Therapies in Mice, Monkeys, Men and Women' on Tuesday by Laurent Desiré

SU57

Abstract

More than half of the congenital non-syndromic deafness cases have a genetic cause that affect specific inner ear cell populations, including sensory hair cells, supporting cells and spiral ganglion neurons, all of which play an important role in the process of hearing. **Adeno-associated virus** (AAVs)-based gene transfer shows great potential for the treatment of hearing loss, as they provide stable gene expression over long period of time and are safe to use. Furthermore, AAV tropism and transgene expression patterns can be fine-tuned through AAV capsid and promotor engineering. To accelerate translation to humans, we now report the **feasibility and efficiency** of an **intracochlear injection through the round window** (RW) for vector delivery in **non-human primates** (NHPs). AAV8-eGFP expressing the enhanced green fluorescent protein (eGFP) was delivered in the cochlea of two-three years old *Macaca fascicularis* by either a **round window injection** (RWI) or a RWI combined with a laser-induced fenestration of the oval window membrane (OWM), also known as **stapedotomy**. A dedicated medical delivery device developed **for clinical use** was employed. Safety was assessed in a GLP-like setting. **Auditory Brainstem Response** (ABRs) and **Distortion Product Otoacoustic Emission** (DPOAE) measurements were performed to assess the hearing function of NHPs before and three weeks after the surgical procedure. Whole mount cochleae were analyzed three weeks post-surgery to evaluate **inner ear morphology** and **AAV8-eGFP tropism**. No clinical, local or systemic findings were reported at the end of the study indicating that the **surgical procedure was well tolerated** and associated with a **good healing process** in the outer, middle and inner ears. The two surgical approaches tested resulted in limited increase of ABR thresholds and decrease of DPOAE amplitudes, which remained contained within the normal hearing threshold range of NHPs. The stapedotomy procedure greatly enhanced the AAV8-eGFP **transduction of inner hair cells** (IHCs) of the organ of Corti, as compared to the RWI procedure. An apico-basal gradient of transduction of IHCs was observed as well as transduction of supporting cells of the greater and lateral epithelial ridges. Most importantly, the intracochlear injection had no impact on the spiral ganglion neuron or IHC survival. This study demonstrated the safety and the feasibility of intracochlear injections in NHPs using our proprietary medical delivery device. Hearing thresholds were generally considered not clinically significant three weeks post-injection given that outer, middle and inner ears were still in a healing state. Thus, AAV8-eGFP efficiently transduces the IHCs of NHPs. AAV8 should be considered as an AAV capsid for inner ear gene therapy in humans. These data motivate future translational studies to evaluate gene therapy for human hearing disorders.

Hearing function assessment in Macaca fascicularis

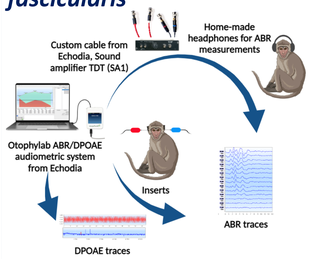

Fig.1: To evaluate the auditory function of NHPs, we implemented reliable methods to record ABRs and DPOAEs using the Otophylab audiometric system developed by Echodia. Inserts placed into the bony part of external auditory canal were initially used to perform close-field ABR measurements. Supra-aural headphones were also developed.

Trans-canal approach, stapedotomy and round window membrane injection (RWI)

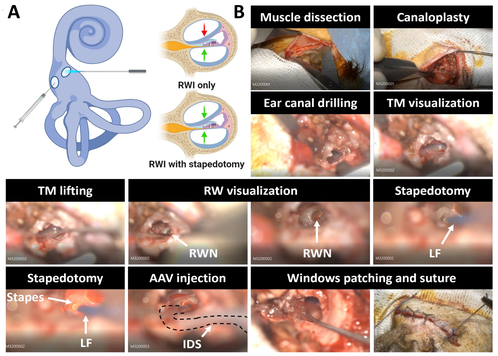

Fig.2: A. Our Surgical approach consists in injecting AAV therapeutic products through the RWM using our proprietary injection device system (IDS) and perforating the foot plate of the stapes (stapedotomy) to alleviate the overpressure in the inner ear and assure a homogenous transduction rate along the tonotopic axis of the cochlea. **B.** The combination of these two techniques well known by ENT surgeons was optimized in NHP studies and is envisioned in human (TM: tympanic membrane, RWM: round window membrane RWN: round window niche, LF: laser fiber).

Enhanced viral-mediated cochlear gene delivery in NHP by combining stapedotomy with RWI

1. Auditory function assessment shows that ABR threshold shifts tend to decrease with time and DPOAE amplitudes improve 3 months after inner ear inoculation

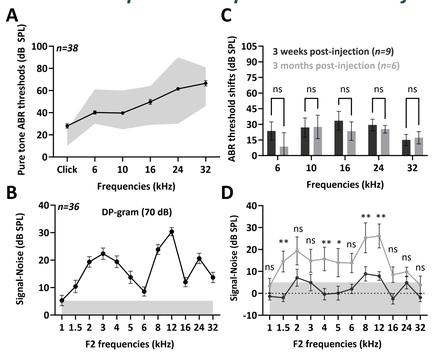

Fig.3: A. ABR pure tone thresholds (dB Sound Pressure Levels (SPL)) were measured before and three weeks post-injection at 6/10/16/24/32 kHz with decreasing intensities from 105 to 10 dB SPL. Gray area in panel **A** shows hearing threshold range measured in naïve NHPs (n=38; mean ± SEM). **B.** DPOAEs (dB) were measured from 1 to 32 kHz using 70 dB stimuli. Signal-noise amplitude (dB SPL) ratios on outer hair cell function were presented. Gray area in panel **B** represents the minimal amplitude for which the DPOAE is considered as present (n=36; mean ± SEM). **C.** ABR threshold shifts (dB SPL) and **D.** signal-noise amplitude (dB SPL) ratios measured from 1 to 32 kHz using 70 dB stimuli three weeks and three-month after a surgical procedure (RWI combined with stapedotomy), show that the inner is healing after the surgery and that DPOAE amplitudes are significantly increasing with time. **A-D.** Error bars represent the standard error of the mean ± SEM. Statistical analyses were performed using Two-way ANOVA multiple comparison within each column tests (**p<0.01, *p<0.1, ns: non-significant).

2. Stapedotomy significantly improves inner hair cell (IHC) transduction rates after RWI and does not affect the organ of Corti architecture

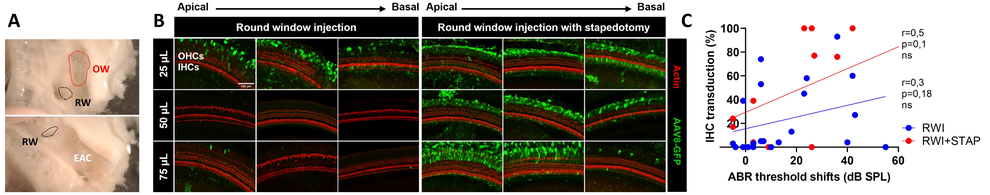

Fig.4: A. Three weeks after AAV8-eGFP injection, temporal bones were collected, fixed and decalcified. Middle ears were analyzed, and no signs of infection or inflammation were observed. **B.** Inner cochleae were fine micro-dissected. Maximum-intensity projections of confocal z-sections from apical, medial, and basal turns of *Macaca fascicularis* cochleae. Whole mount cochleae were immunolabelled using an anti-GFP antibody (green) to evaluate the transduction efficiency after RWI combined or combined with stapedotomy procedure. An actin marker (red) was used to highlight and quantify hair cell (HC) loss. Uninjected cochleae were used as control. Scale bar: 100 µm. **C.** Percentage of IHC transduction was quantified. ABR threshold shifts measured at 6 and 10 kHz were averaged and represent stimulation of the middle turn of the cochlea based on cochleogram analysis. Similarly, ABR threshold shifts at 16, 24 and 32 kHz were averaged and represent stimulations of the mid to base turns of the cochlea. IHC transduction rates were correlated to ABR threshold shifts for each animal. Individual data are represented as blue dots for RWI (n=11) or as red dots for RWI combined with stapedotomy (n=6). Statistical analyses were performed using Spearman correlation coefficient and linear regression (ns: non-significant). As a conclusion, combining RWI with a stapedotomy procedure significantly improves IHC transduction rates without increasing ABR hearing thresholds.

3. Stapedotomy combined with RWI does not lead to spiral ganglion neuron or hair cell loss in the inner ear

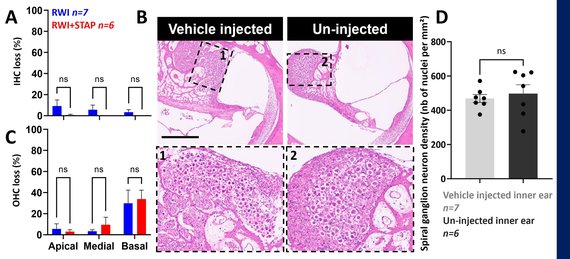

Fig.5: A, B. HC loss was quantified from X60 acquisitions using ImageJ shown in Fig. 4. Error bars represent the standard error of the mean ± SEM. RWI are represented with blue bars (n=7), RWI combined with stapedotomy with red bars (n=6)**.** Statistical analyses were performed by T-tests (p>0.1, ns: non-significant). **C.** Sections of NHPs temporal bones injected with vehicle following a RWI in combination with stapedotomy were processed 3 months after RWI for hematoxylin/eosin staining to assess the structure of the spiral ganglion and organ of Corti . Uninjected inner ears were used as controls. **D.** Quantification of the spiral ganglion neuron density was performed using ImageJ. Error bars represent the standard error of the mean ±SEM. Vehicle injected ear represented as a light gray bar n=7, and uninjected ear as a dark gray bar n=6)**.** Statistical analysis was performed using student T-tests (ns: non-significant). Scale bar=600 mm

4. Little to no impact of pre-existing anti-AAV8 capsid Neutralizing Antibodies (Nabs) on IHC transduction rates in the NHP inner ear

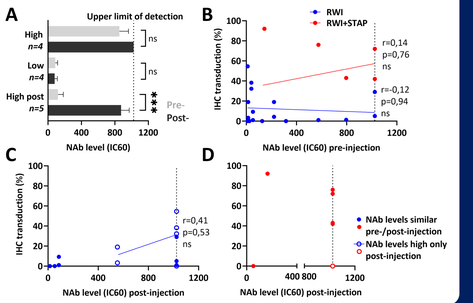

Fig.6: A-D, Blood samples were collected from NHP before the surgical procedure and 3 weeks post- AAV8-eGFP injection. AAV-neutralizing antibodies levels were determined using an AAV-binding assay. AAV8 transduction inhibition titers were determined as the reciprocal dilution of plasma interpolated at the titer cut point set 61.60% of transduction through linear regression. AAV8-luc vector at the determined MOI was incubated to varying dilutions of serum samples for 1 hour±15 min at 35-38°C. The resulting complex of AAV8-luc vector and serum samples containing neutralizing antibodies (NAbs) was then applied to HEK-293 cells for 48±4 hours. **A**, The neutralizing 60% binding (IC60) values were then calculated, as represented by the antibody dilutions needed to neutralize 60% of the input vector. Animals considered with NAb titers high pre- and post-injection, low pre- and post-injection, or high only post-injection, were averaged (data are expressed as mean percent neutralized vector ± SEM). **B.** IHC transduction rates were correlated to pre-injection IC60 for each animal. Data were represented in order to assess a correlation between IHC transduction and NAb titers, **B-D.** in function of the surgical procedure or **C, D,** in function of the NAb status after the surgery. **B-D** Individual data are represented as blue dots for RWI (n=14) or as red dots for RWI combined with stapedotomy (n=4).

Conclusions

- Audiometric equipment and protocols were optimized in order to efficiently assess the auditory function of NHP through ABR (Click, 6-32kHz) and DPOAEs measurements
- A medical injection device was developed based on our NHP studies and has been approved for use in clinical trials (France and UK).
- Stapedotomy and RWI leads to efficient and homogenous transduction rate of the IHC without affecting the inner ear morphology nor spiral ganglion neuron nor HC loss. Thus, the procedure does not lead to high ABR threshold shifts or DPOAE amplitude deficit
- It appears that high circulating anti-AAV Nabs in NHP is not a burden and is not associated with a decrease of IHC transduction efficiency in the inner ear.

Acknowledgements

The authors would like to thank Théo Trope from 34 bis Fablab for his help with the design of the supra-aural headphones. Studies were performed at Charles River laboratory facilities and authors would like to thank Clémentine Chalmey, Mathieu Laugier, Sebastien Gremont, Sylvie Bonavaud and Stephanie Bergère. The authors would like to thank Montpellier Ressources Imagerie plateform (MRI, CNRS).