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Pharmacokinetics and tissue distribution of neurotrophin 3 after intracochlear delivery

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Short title: Distribution and clearance of Ntf3 in the cochlea

Abstract

Neurotrophin therapy has potential to reverse some forms of hearing loss. However, cochlear pharmacokinetic studies are challenging due to small fluid volumes. Here a radioactive tracer was used to determine neurotrophin-3 retention, distribution and clearance after intracochlear administration. ^{125}I-neurotrophin-3 was injected into guinea pig cochleae using a sealed injection technique comparing dosing volumes, rates and concentrations up to 750 µg/mL. Retention was measured by whole-cochlea gamma counts at five time point while distribution and clearance were assessed by autoradiography. Smaller injection volumes and higher concentrations correlated with higher retention of neurotrophin-3. Distribution of neurotrophin-3 was widespread throughout the cochlear tissue, decreasing in concentration from base to apex. Tissue distribution was non-uniform, with greatest density in cells lining the scala tympani and lower density in neural target tissue. The time constant for clearance of neurotrophin-3 from cochlear tissues was 38 hours but neurotrophin-3 remained detectable for at least 2 weeks. Neurotrophin-3 was evident in the semi-circular canals and minor spread to the contralateral cochlea. This study is the first comprehensive evaluation of the disposition profile for a protein therapy in the cochlea. The findings and methods in this study will provide valuable guidance for the development of protein therapies for hearing loss.

Key words

Neurotrophin-3, cochlea, drug delivery, radiolabel, clearance, distribution, pharmacokinetics, hearing loss

Background

Hearing loss represents a major public health problem worldwide with an estimated 466 million people suffering from this sensory disorder (World Health Organization, 2018). Furthermore, as the population ages, the prevalence of hearing loss is expected to rise. Hearing impairment can cause delays in the development of speech, cognitive and social skills in children[1] and impact on work and social activities in adults, with links to depression and the incidence of dementia[2-4]. Sensorineural hearing loss is the most common form of hearing loss, characterized by damaged sensory cells within the inner ear (cochlea). At present there are no drug therapies to treat hearing loss. The only interventions for sensorineural hearing loss are devices such as hearing aids and cochlear implants which do not address the underlying cause of the hearing loss. Benefits provided by devices are typically varied and generally poor in challenging acoustic situations, such as noisy backgrounds or when listening to music[5]. The need for a therapeutic intervention to treat hearing loss is high and the most effective strategy is to repair cochlear damage before it becomes a debilitating condition.

The sensory cells that detect sound (hair cells) and the spiral ganglion neurons that convey the information to the brain are sensitive to damage from excessive noise exposure[6, 7], ototoxic drugs such as antibiotics and ageing[8]. Following significant damage, hair cells die via apoptosis with no evidence of spontaneous regeneration in mammals. However, experimental animal studies indicate that the synapse between the hair cell and the spiral ganglion neuron can be protected and repaired by increasing the intracochlear level of neurotrophin 3 (Ntf3) or brain-derived neurotrophic factor (BDNF) after damage[9-11]. Furthermore, introduction of exogenous neurotrophins to the cochlea preserves spiral ganglion neurons and also promotes peripheral nerve fibre resprouting following hearing loss, even in the absence of hair cells[12], with implications for reduced thresholds of neural activation when used in conjunction with cochlear implantation[13].

Different strategies to deliver neurotrophins to the cochlea have been extensively studied. Most effective for spiral ganglion neuron protection and fibre regeneration is direct and continuous

delivery of high concentrations of neurotrophins via a mini-pump connected to the cochlea by a cannula sealed into the cochlear wall[14-18]. This type of direct drug delivery would be most useful in scenarios where the cochlea is already being accessed, for example, during cochlear implantation. Because surgical breach of the cochlear bony wall does carry some risk of sensory cell damage, middle ear delivery options are also being developed whereby therapeutics can diffuse into the cochlea via two semi-permeable membranes (the round and oval windows)[19, 20]. However, the drug levels achieved in cochlear fluids are significantly reduced with this method. It is necessary to understand the pharmacokinetics of Ntf3 in the cochlea following the different delivery techniques to compare the effectiveness of each strategy. In particular, the retention, clearance rate and distribution are of interest to determine how much drug is available to the target cells, the spiral ganglion neurons, and for how long.

Pharmacokinetic analysis in the cochlea is complicated by the small fluid volumes and the fact that sampling can itself affect the outcomes by inducing non-physiologic fluid flow where the cochlear perilymph fluid is contaminated with cerebrospinal fluid (CSF)[21]. New sampling methods have been devised to minimise CSF contamination[22, 23] but fluid sampling can still only show what is present in the cochlear fluids; there is no way to accurately determine how much drug is within the cochlear tissue and in what proportions. This study addresses this critical aspect by using radiolabelled Ntf3 to visualise and quantify the distribution of Ntf3 in the cochlear tissue, measure clearance and examine the relative distribution of the Ntf3 in the cochlea. Radiolabelling of Ntf3 has been previously used to show the Ntf3 distribution throughout the cochlea in cellular detail after direct administration to the cochlear fluids[24, 25]. Ntf3 was found to spread to all cochlear turns and to have a relatively slow clearance from the tissues. However, the fluid component was not assessed in the previous studies, and dosing rate, volume and buffer were not optimal. Here we extend these findings by examining the retention, clearance and distribution of Ntf3 in the cochlea after local intracochlear delivery using a fully sealed system. The technique has the potential to enable direct comparison of different drug delivery strategies to the cochlea and aid development of drug therapies for hearing loss.

METHODS

Neurotrophin labelling

Human Ntf3 (PeproTech; 3-4.5 μg) was radio-labelled with 0.25 mCi Na ^{125}I (ICN Biomedicals Australasia) using the Chloramine-T method (ProSearch International) [26]. Unbound ^{125}I was separated from Ntf3-bound ^{125}I by Biogel P6DG column chromatography. Incorporation of ^{125}I into Ntf3 was between 55 and 91%. Specific activity was 46-93 μCi/ug. The radio-labelled Ntf3 was supplied in phosphate buffer containing 0.25% (w/v) BSA and 0.1% (w/v) sodium azide. Five different batches were used in the study.

^{125}I Ntf3 was purified and buffer exchanged to artificial perilymph using an Amicon Ultra-4 10K filtration unit (for Batches 1-4) or Amicon Ultra-2 3K filtration unit for Batch 5. The filter was pre-coated for 20 minutes in a 1% (w/v) BSA solution in PBS which was then centrifuged through for 2 minutes and discarded. One millilitre of ^{125}I Ntf3 was added and centrifuged though the filter at 5500 *g* for 10 minutes. The ultrafiltrate was removed and set aside for gamma counting. Artificial perilymph (125 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2mM $MgCl_2$, 0.75 mM NaH_2PO_4, 5 mM glucose, pH 7.4) was added to the concentrated ^{125}I Ntf3 and this was centrifuged through the filter unit. This process was repeated. The final ^{125}I Ntf3 sample was collected, the volume measured and a sample taken for gamma counting.

Concentration of ^{125}I Ntf3 was estimated using specific activity, assuming no losses. Following purification and buffer exchange, concentration was adjusted to 0.1 μg/ml, 0.5 μg/ml, 0.9 μg/ml or 1.5 μg/ml. For the dosing concentration study, artificial perilymph buffer or unlabelled Ntf3 concentrate at 150 μg/mL and 1500 μg/mL, respectively, was added to 1.5 μg/ml radiolabelled Ntf3 at a 1:1 volume ratio to achieve final concentrations of 0.75 μg/ml ^{125}I Ntf3 and 75 μg/ml or 750 μg/ml unlabelled Ntf3.

Injection into guinea pig cochleae

Thirty one tri-colour pigmented guinea pigs were used in this study. The use and care of the experimental animals in this study were approved by St Vincent's Hospital (Melbourne) Animal Ethics Committee (#007/17) and follows the Guidelines to Promote the Wellbeing of Animals used for Scientific Purposes (2013), the NHMRC Code for Care and Use of Animals for Scientific Purposes (8th edition, 2013) and the Prevention of Cruelty to Animals Amendment Act (2015).

(i) Cannula preparation

Cannulas were prepared from polyethylene tubing (OD 0.96 mm, ID 0.58 mm) attached to a blunt needle at one end and a 3 cm polyimide tube (OD 0.16 mm, ID 0.12 mm) at the other end with the joins sealed by silicone. A tapered silicone plug was created 2 mm from the tip to help seal the polyimide tube into the cochlea during injection. Prior to injection, the cannula was first loaded with liquid paraffin then attached to a 10 μL Hamilton syringe connected to a rate-controlled microdrive unit. Due to the potential of the Ntf3 protein to stick to the cannula, the cannula was pre-coated with ^{125}I Ntf3 solution by drawing up the desired volume, leaving for 20 minutes and discarding the solution prior to loading.

A sample of the injected dose equal to the desired injection volume was first dispensed into a radioimmunoassay tube containing 50 μL water for gamma measurement prior to drawing up the same volume again (plus an extra 10%) for injection into a guinea pig cochlea.

(ii) Injection

Guinea pigs were anaesthetised with an intramuscular injection of a ketamine/xylazine mixture (60 mg/kg ketamine and 4 mg/kg xylazine). Under aseptic conditions, a post-auricular incision was made and the underlying muscles were gently separated to expose the ventral bulla. The bulla was opened with a #14 diamond burr to visualise the lower basal turn of the cochlea. The membrane over the

basal turn of the cochlea was gently retracted and a cochleostomy was performed with a fine, sharpened metal probe. The polyimide tip of the prepared cannula was inserted into the cochlea up to the silicon plug with the tapered end inserted until a seal was formed. Further sealing was provided via superglue. The cannula was also secured to the bulla with superglue to ensure stability of the delivery system. When the glue had dried, the desired volume of ^{125}I Ntf3 was injected into the cochlea at the set rate (0.2 μL/min or 2.5 μL/min). When complete, the polyimide part of the cannula was simultaneously sealed and cut using a cautery (therefore a small part of the polyimide remained in the cochlea to maintain the cochlear seal). The bulla was sealed with dental cement and the muscle and skin layers were closed separately with 3-0 Vicryl and surgical metal clips. For each study cohort, injections were performed within 10 days of each other with radioactive decay accounted for in all measurements.

Cochlear extraction

Between 0 and 336 hours post-injection, guinea pigs were given a lethal dose of pentobarbitone. When breathing had stopped, the animals were intracardially perfused with 400 mL 0.9% (w/v) saline containing 0.1% (w/v) heparin sodium and 0.025% (w/v) sodium nitrite. This was followed by intracardial perfusion with 400 mL neutral buffered formalin (NBF). The bulla was dissected from the temporal bones. A small hole was made in the bulla and the remaining polyimide delivery tube was visually confirmed to be secure in the cochlea. The rest of the bulla was carefully dissected away. Fluid loss from the cochlea was not visible but this final part of the dissection was performed over the radioimmunoassay tube so that any fluid loss would be captured for gamma reading. The cochlea, with semi-circular canals still attached (as it is difficult to reliably dissect the cochlea away from the semi-circular canals without compromising fluid integrity), was placed in a radioimmunoassay tube containing 0.5 mL formalin for gamma counting. Study time points were taken at the point of intracardial perfusion of NBF with the shortest time point therefore being the time taken to prepare the animal for perfusion (15 min).

Gamma measurements

All gamma measurements (injected dose samples and dissected cochleae) were obtained within 24 hours of the perfusion using a Perkin Elmer WIZARD automatic gamma counter and measured over 1 minute.

Histology

After gamma counting, the tissue was prepared for sectioning. Cochleae and semi-circular canals were decalcified in 10% (w/v) ethylenediaminetetraacetic acid over 1-2 weeks, oriented and frozen in optimal cutting temperature (OCT) compound, sectioned through the mid-modiolar plane at 12 μm and placed onto Superfrost slides (3 sections per slide). Slides were stored at -20^{o}C until use.

Autoradiography and imaging

(i) Standards preparation: Whatman filter paper disks (5 mm diameter) were prepared and numbered 1-10. Each disk was inoculated with a 1/3 serial dilution of ^{125}I NaI and PAGE blue dye in a solution containing 0.1 M NaI, 0.1 M sodium metabisulphite (pH 10), 30% (v/v) ethanol and 3.1 mg/ml PAGE blue. Three microliters of each dilution was pipetted onto the disks, with 1 being the most concentrated at 0.22 μCi. The disks were aligned in straight rows onto a strip of sticky tape which was then adhered to a glass microslide for exposure to film.

(ii) Film: Defrosted and dried slides (3 per cochlea), as well as the radiological standards slides, were exposed to Biomax MR film (Kodak) and developed after 1 week, 2 weeks and 4 weeks in a medical film processor (SRX-101A; Konica Minolta Medical and Graphic, Inc). Developed films were scanned using an Epson v800 photo scanner (grayscale, 16-bit, 2400 dpi with no compression). For each series (1 week, 2 week or 4 week exposure), a calibration was performed on the prepared radiological standards in ImageJ using the Rodbard function. Area, mean grey value, standard

deviation and integrated density measurements were then taken of the whole cochlear section by tracing around the section using the freehand drawing tool. Background measurements were obtained which were subtracted from the cochlear measurements. In cases where strong signal was coming from an intrascalar source (e.g. debris and fibrous tissue from surgery or artefact), the debris/artefact was measured independently and subtracted from the whole section measurements.

(iii) Emulsion: Slides were defrosted, dried and placed in water to remove the OCT. Slides were then dehydrated through a series of ethanol (70%, 90% and twice at 100% v/v ethanol) and then air dried. Slides were coated on the front only (back-to-back coating technique) with Kodak NTB emulsion at 42^{o}C. Coated slides were dried vertically for 5 minutes and then placed in a light-tight box for 1 hour at room temperature. Anhydrous silica was then added to the boxes before the slides were placed at 4^{o}C for between 1 and 4 weeks.

Some slides were pre-stained with H&E prior to coating with emulsion. A standard H&E protocol was used, with the incubation times doubled such that the sections were over-stained. This was to compensate for loss of staining during post-processing.

After the set exposure period of 1-4 weeks, the slides were brought to room temperature for 1 hour. Under darkroom conditions slides were placed in Dektol developer (Kodak; diluted 1:1 with water) for 5 minutes, a water stop bath for 30 seconds and fixer (Kodak) for 10 minutes. Slides were rinsed in running water for 15 minutes prior to dehydration through graded ethanol (90% and 100% v/v), xylene and then coverslipped in DPX.

Images of the entire cochlear mid-modiolar cross section as well as the lower basal and upper basal turns were taken using the 2.5x and 10x objectives on a Zeiss Axio Imager M2 upright microscope. Up to 10 images were stitched together using the Zen software stitching function, with 5% overlap. All images were taken using identical light and exposure settings. Images were analysed using ImageJ software. Mean gray values for an 8-bit image (0-255) were calibrated to an optical density value between 0 (white) and 3.05 (black) using a Kodak No.3 Calibrated Step Tablet and the Rodbard

function (r^2=0.9992). Calibrated optical density, integrated density and area were measured for each whole turn (tissue only, fluid filled chambers excluded). Calibrated optical density, integrated density and area were measured for specific basal turn regions of interest (basilar membrane, spiral limbus, spiral ligament, Rosenthal's canal, peripheral nerve fibre and cells lining scala tympani). Background was measured from a part of the slide containing tissue that was negative for the radiolabel signal.

Analysis of results

Retention of ^{125}I Ntf3 in the cochlea is expressed as a percentage of the dose injected. Data is presented as individual points or as mean ± standard error of the mean with statistical comparison of groups carried out with an ANOVA. A regression analysis was performed on data to examine the effect of concentration on retention of ^{125}I Ntf3. Curve fitting analysis was performed to compare clearance over time. Autoradiographic film analysis was performed in ImageJ with calibration as described. Background was subtracted and the data was normalised for comparison of different exposure times. A total of 9 sections were measured for each cochlea. Analysis of emulsion coated slides was performed in ImageJ as described with data expressed as relative integrated density with background subtracted.

RESULTS

Higher perilymph and tissue retention are achieved with smaller dosing volumes

^{125}I Ntf3 (0.1 µg/mL) was injected into guinea pig cochleae at 1 µL, 5 µL or 20 µL dosing volumes (total dose 0.1, 0.5 or 40 ng) at rate of 0.2 µL/min or 2.5 µL/min (n=13). Four samples were in artificial perilymph (AP) and nine samples were in phosphate buffer (PB). Gamma counts of the cochleae (semi-circular canals included) at a four hour time point were used to compare the retention of the ^{125}I Ntf3 dose, expressed as a percentage of the gamma measurement of the injected dose.

Figure 1A shows retention in cochlear tissue for the different volumes injected. The measurements were made following a 24 hour incubation in fixative during which the perilymphatic fluid and loosely bound ^{125}I Ntf3 would have dispersed from the cochlear tissue and was measured separately as 'perilymph' in some cases. Regression analysis of the data revealed that volume had a significant effect on retention (R^2=0.59, p=0.002). Following slow rate injection of 1 µL, retention in cochlear tissue was 15.8% ± 2.6% (0.02 ng) of the injected dose. For 5 µL slow rate injection the average retention was 7.0 ± 1.1% (0.03 ng). In contrast, only 1.9% of the starting material (0.04 ng) remained in the cochlear tissue following the 20 µL injection at the fast rate. Measurement of the perilymph from cochleae in the 5 µL slow rate injection group revealed that 46% of the total dose was in the perilymph at the 4 hour time point, boosting the total dose in the cochlea to 18% (0.09 ng) of the injected material. In the fast rate groups, the perilymph component of the retained cochlear dose was lower at 29%.

The average tissue retention was similar for dosing solutions prepared in phosphate buffer and artificial perilymph: 5.8% ± 1.5% and 8.8% ± 0.5%, respectively. The 2.5 µL/min injection rate group displayed greater variability in retention (6.9% ± 3.1%) compared to the 0.2 µL/min rate group (7.0% ± 1.1%) for a 5 µL dosing volume at 4 hours post-injection (Figure 1A).

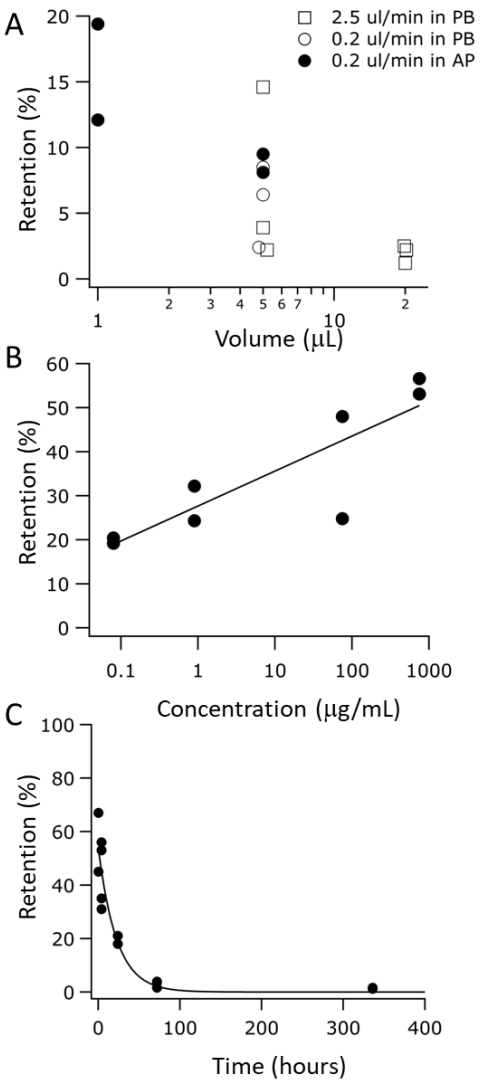

Figure 1: Effect of dosing volume, concentration and time on retention (percentage of the injected ^{125}I Ntf3 dose remaining in the cochlea). (A) Retention at 4 hours in cochlear tissue only following injection of different volumes of 0.1 µg/mL ^{125}I Ntf3. The symbols show the different rates of injection and the buffer used (n=13). PB=phosphate buffered saline; AP=artificial perilymph. Regression analysis between retention and log(Volume) was fitted to the data. Retention=14.8-(10.4*LogVolume), R^2=0.59, p=0.002, n=13. **(B)** Retention in perilymph and tissue combined at 4 hours post-injection of 5 µL ^{125}I Ntf3 at different concentrations. The regression between Retention and log(Concentration) was fitted to the data: Retention = 27.7 + 7.9*log(Concentration), R^2=0.7, p=0.006. **(C)** Retention in perilymph and tissue combined over a time series ranging from 15 minutes to 2 weeks following injection of 5 µL of a total dose of 3.75 µg Ntf3 (unlabelled Ntf3 at 750 µg/mL

and radiolabelled Ntf3 at 0.75 µg/mL). There was an exponential decrease in Ntf3 retained in the cochlea over time which can be expressed as Retention = 54.11*exp(-Time/22.4019).

Greater retention with higher dosing concentrations

Cochleae were injected with 5 µL of ^{125}I Ntf3 at 4 different concentrations (0.08 µg/mL, 0.9 µg/mL, 75 µg/mL or 750 µg/mL). The latter two dosing solutions consisted of a 1:100 or 1:1000 ratio of labelled to unlabelled Ntf3. i.e. 0.75 µg/mL ^{125}I Ntf3 mixed with 75 µg/mL or 750 µg/mL unlabelled Ntf3. The rate of injection was 0.2 µL/min (n=8). Whole-cochlea gamma measurements were collected 4 hours after injection, this time combining cochlear tissue and perilymph. There was a significant correlation between percentage retention and concentration of the dosing solution at the 4 hour time point (R^2=0.7, p=0.006) (Figure 1B). A dosing concentration of 0.08 µg/mL Ntf3 resulted in an average retention of 19.8% ± 0.41% (or 79 pg) of the starting material in the cochlear tissues and perilymph while a dosing concentration of 751 µg/mL Ntf3 resulted in an average retention of 54.8% ± 1.2% (or 2.1 µg).

Exponential clearance of Ntf3 after intracochlear delivery

To determine the clearance of Ntf3 from the cochlea over time, cochleae (n=12) were injected with 5 µL of the Ntf3 dosing solution (a mixture of 0.75 µg/mL ^{125}I Ntf3 and 750 µg/mL unlabelled Ntf3; total dose 3.75 µg) at a rate of 0.2 µL/min with five treatment durations (15 min to 2 weeks). Clearance was determined from whole-cochlea gamma measurements of both cochlear fluids and tissue. A single exponential was fit to the retention data (Figure 1C). There was significant bulk loss of the radiolabelled Ntf3 during or just after injection (calculation of Time 0 from the curve predicts retention of < 60% of the dosing material) and the time constant (the time taken to drop to ~36% of the current value) for clearance from the whole cochlea was approximately 22 hours. Although most of the injected Ntf3 had been eliminated from the cochlea by 3 days (3.1% of the injected dose

remaining; 0.12 µg), gamma signal from the radiolabel persisted within the cochlea at the 2 week time point where the signal from the residual ^{125}I Ntf3 was 40 times less compared to the 15 minute time point with just 1.4% of the injected dose detected in the cochlea. The absolute level of ^{125}I Ntf3 signal detected at the 2 week time point averaged 4029 cpm, which was 54 times the detection limit of 75 cpm (defined as 3x noise floor measurements – average 25 cpm) and 16 times the quantification limit of 250 cpm (defined as 10x noise floor measurements) .

Uptake in all cochlear turns with slow tissue clearance

Sections of cochleae from Figure 1C were exposed to BioMax MR Film in order to analyse the uptake and clearance of ^{125}I Ntf3 within the cochlear tissues. The ^{125}I signal from the entire cross-section was measured and expressed as integrated density (Density*area) for cochleae in the 5 time points. In the 15 minute, 4 hour and 1 day groups, the radiolabelled Ntf3 signal was detectable in all turns of the cochleae but was strongest in Turns 1 and 2 (lower basal and upper basal turns) (Figure 2A). By 3 days the signal in the apical turns was no longer detectable (Figure 2B). A single exponential was fitted to the normalised integrated density data (Figure 2E). The time constant for clearance of ^{125}I Ntf3 signal from cochlear tissue was approximately 38 hours, modelling the cochlear tissue as a single compartment. A linear regression between integrated density and log(time) demonstrated a significant effect of time on the amount of Ntf3 in the cochlear sections ($R^2 = 0.73$, $p<0.001$). Signal to background ratios ranged from 12.3 fold for the 15 minute group to 1.15 fold for the 2 week group. Signal in the 2 week group was over 16 times lower than to the 15 minute group and was detectable only in the basal turn. Low level signal in the contralateral (non-injected) cochlea was observed in one out of two animals from the 15 minute group (Figure 2C).

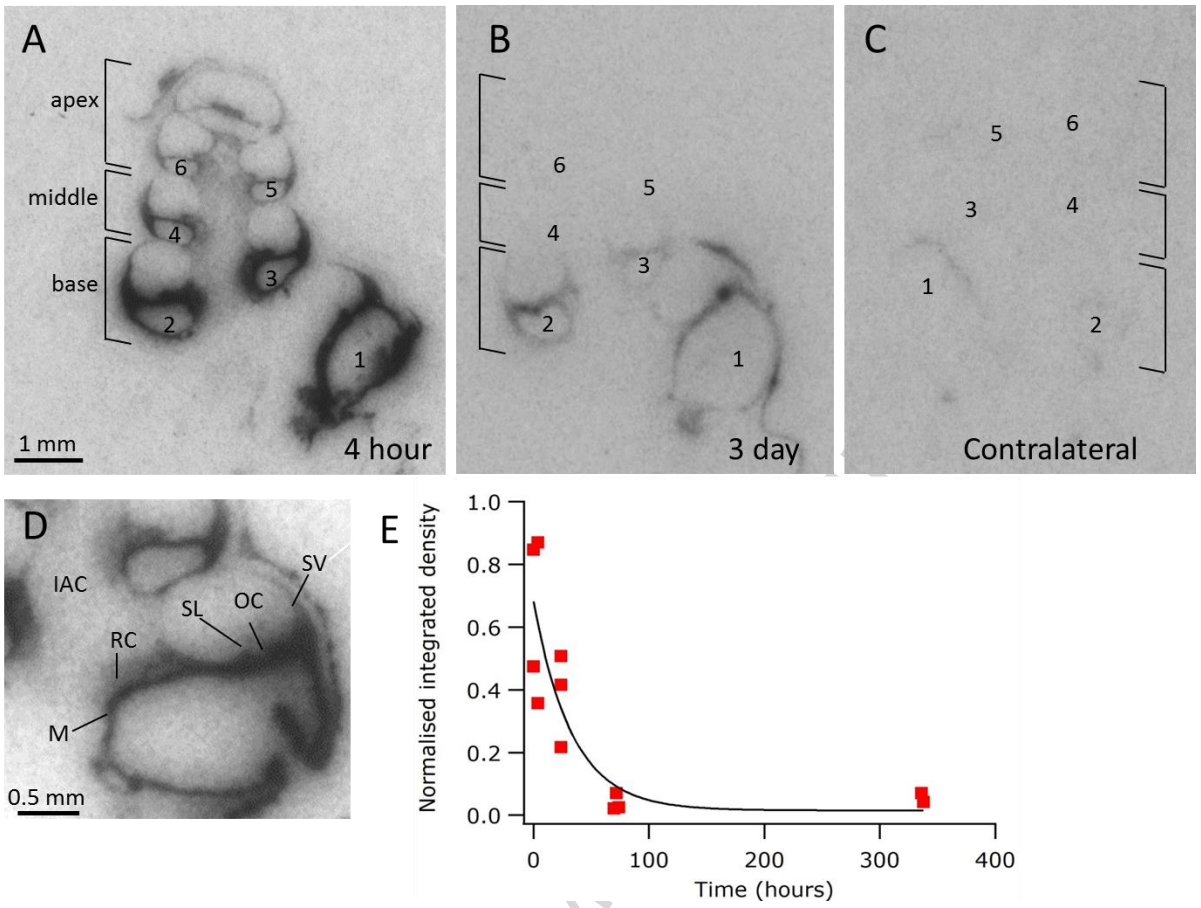

Figure 2: Distribution of ^{125}I Ntf3 in cochlear tissue and clearance over time. Radiological signal from cochlear sections exposed to Kodak MAR Film for 4 weeks. **(A)** 4 hour group, **(B)** 3 day group, **(C)** Contralateral cochlea from 15 minute group. In **(A-C)** the scala tympani of each turn is labelled 1-6. Scale bar applies to all images. **(D)** Detail of Turn 1 where IAC=internal auditory canal, RC=Rosenthal's canal, SL=spiral limbus, OC=organ of Corti, SV=stria vascularis and M=mesothelial cells lining scala tympani. **(E)** Analysis of radiological signal (film). Normalised integrated density (background corrected) of the radiolabelled Ntf3 signal in the entire cochlear section in each group (n=2-3 animals per time point). There was an exponential decrease in Ntf3 retained in the cochlear tissue over time which can be expressed as Integrated Density = 0.68*exp(-Time/37.7566).

Non-uniform tissue distribution

Autoradiographic emulsion was used for histological analysis of distribution throughout the cochlea. Signal from ^{125}I Ntf3 was detectable in neural tissues such as Rosenthal's canal which houses the spiral ganglion neuron cell bodies and the internal auditory canal which contains axons from the spiral ganglion neurons projecting towards the central auditory structures (Figure 3; see also Figure 2). There was also signal in non-neural tissues such as the medial and lateral bony tissue of the cochlea, the organ of Corti which houses the sensory hair cells, spiral ligament, spiral limbus, stria vascularis and mesothelial cells lining the perilymphatic spaces. ^{125}I Ntf3 also appeared to accumulate in the endothelial cells lining the scala media including the inner spiral sulcus (Figure 3). The ^{125}I signal was visually strongest in the 15 minute and 4 hour groups but was still present in cochleae in the 2 week group, mainly in the basilar membrane region.

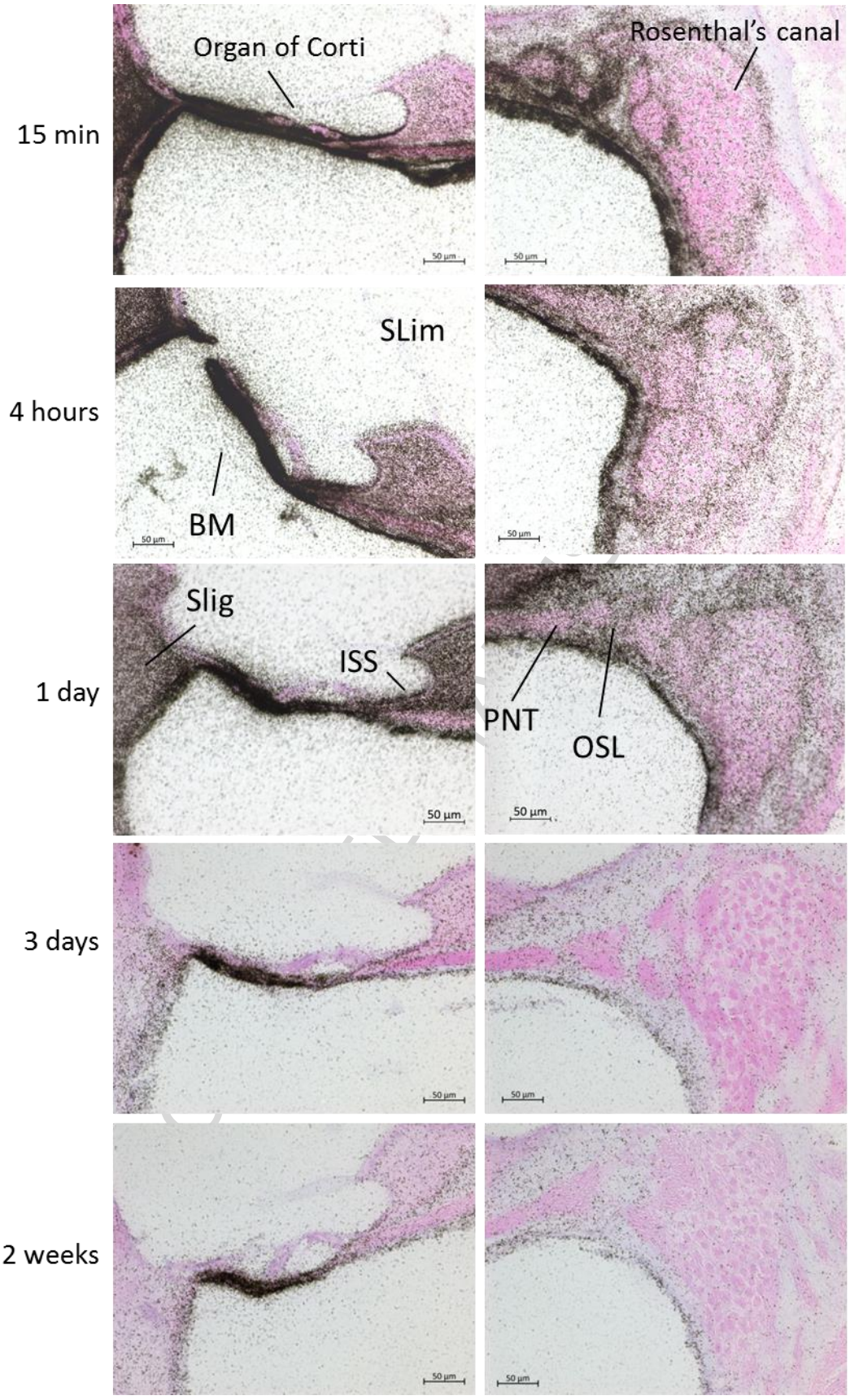

Figure 3: Distribution and clearance of ^{125}I Ntf3 from cochlear tissue over time. H&E stained autoradiographs of the cochlear upper basal turn from each group with the left column showing images of the organ of Corti region (OC) and the right column showing images of the Rosenthal's canal (RC) region. In all groups ^{125}I Ntf3 signal was strongest in the basilar membrane (BM) and mesothelial cells lining the perilymphatic chamber into which the dosing material was injected. In the 15 minute, 4 hour and 1 day groups there were also high levels of ^{125}I signal in the spiral ligament (SLig), osseous spiral lamina (OSL), the peripheral nerve tract (PNT) that runs through the OSL and the spiral limbus (SLim). Levels of ^{125}I Ntf3 in Rosenthal's canal were lower than surrounding tissue. The signal strength was reduced in the 3 day and 2 week groups, except for the BM which remained strong in all groups. ISS-inner spiral sulcus

Basal to apical tissue gradient at all time points

Distribution of ^{125}I Ntf3 along the length of the spiralling cochlear tissue was examined turn by turn using unstained mid-modiolar emulsion-based autoradiographs. Turns were labelled 1-6, with turns 1 and 2 representing lower basal and upper basal turns, respectively, as they appear in cross section (Figure 4A). Integrated density was measured in the outlined tissue excluding the fluid filled spaces as shown in *Figure 4*A. There was an overall main effect of cochlear turn and treatment group with regards to the integrated density per mm^2 ($p<0.05$; two way ANOVA). Multiple comparisons using the Holm-Sidak method revealed that for all time points the basal turn of the cochlea (Turns 1 and 2 combined) had a significantly higher ^{125}I Ntf3 signal density of the radiolabel compared to the apical turns (Turns 5 and 6). Also, the 15 minute group had significantly higher ^{125}I Ntf3 signal density compared to the 3 day and 2 week groups, which did not have signal detectable above background measurements turns 1 and 2 (*Figure 4*B; ($p<0.05$; two-way ANOVA). The 4 hour group did not follow the trend of the other groups, with the turn 1 measurement being half the value of turn 2. This was confirmed by visual inspection of the autoradiographs. Although signal for the radiolabel was

observed in one contralateral cochlea on the film autoradiographs (see *Figure 2*), the signal was not detectable by measurement or by visual inspection of sections coated with emulsion.

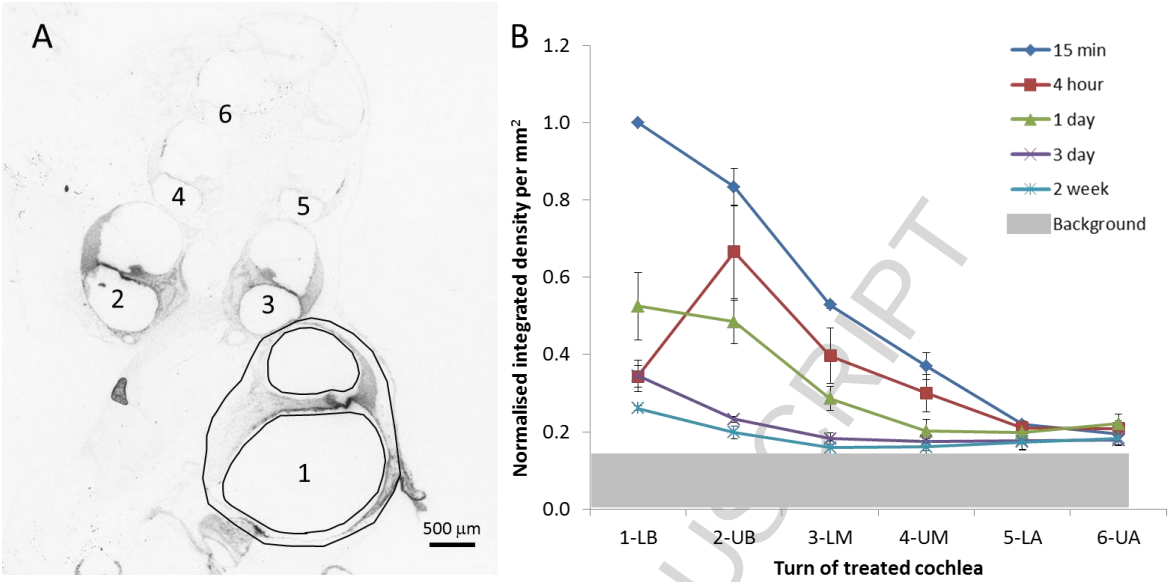

Figure 4: Basal to apical gradient of ^{125}I Ntf3 in the cochlea. Radiological signal from cochlear sections exposed to autoradiographic emulsion. **A** Unstained autoradiograph showing the ^{125}I signal from a mid-modiolar cochlear section from the 1 day group. A region of interest has been drawn around the tissue of the lower basal turn (Turn 1) as an example of how concentration (Integrated density) of the ^{125}I Ntf3 signal was quantified. This was repeated for 6 cochlear turns. **B** Integrated density per mm^2 of radiolabel in each turn of the injected cochlea measured from cochlear sections coated with emulsion. Error bars show the standard error of the mean. L-lower, U-upper, B-basal, M-middle, A-apical

Non-uniform tissue uptake of ^{125}I Ntf3

As seen in the H&E images, the ^{125}I autoradiographic signal strength differed between different cochlear tissues. The signal for ^{125}I was particularly strong in the mesothelial cells lining the scala tympani, including the basilar membrane, for all groups. Lower signal density was detected in tissues

such as the spiral ligament and in the peripheral nerve fibres while the lowest signal density was detected in the spiral limbus and Rosenthal's canal (Figure 5).

Relative intracochlear Ntf3 integrated densities were compared group by segmenting different regions of interest within the lower and upper basal turns of the cochlea (Turns 1 and 2) as described in Figure 5C. The region with the highest concentration of the radiolabel was the basilar membrane. At 4 hours post-injection, the integrated density was 4.5 times higher than the average of other tissues combined in the lower basal turn (Figure 5D). The basilar membrane signal remained high over all time points while the signal in the other tissues tended to reduce over time. The neural tissue (Rosenthal's canal and nerve fibres) had a moderate density of Ntf3 signal that was similar to surrounding non-neural tissue (spiral ligament and spiral limbus) (Figure 5D). Measurement of contralateral sections revealed that none of the sections contained radiolabel signal that was above the level of background (data not shown).

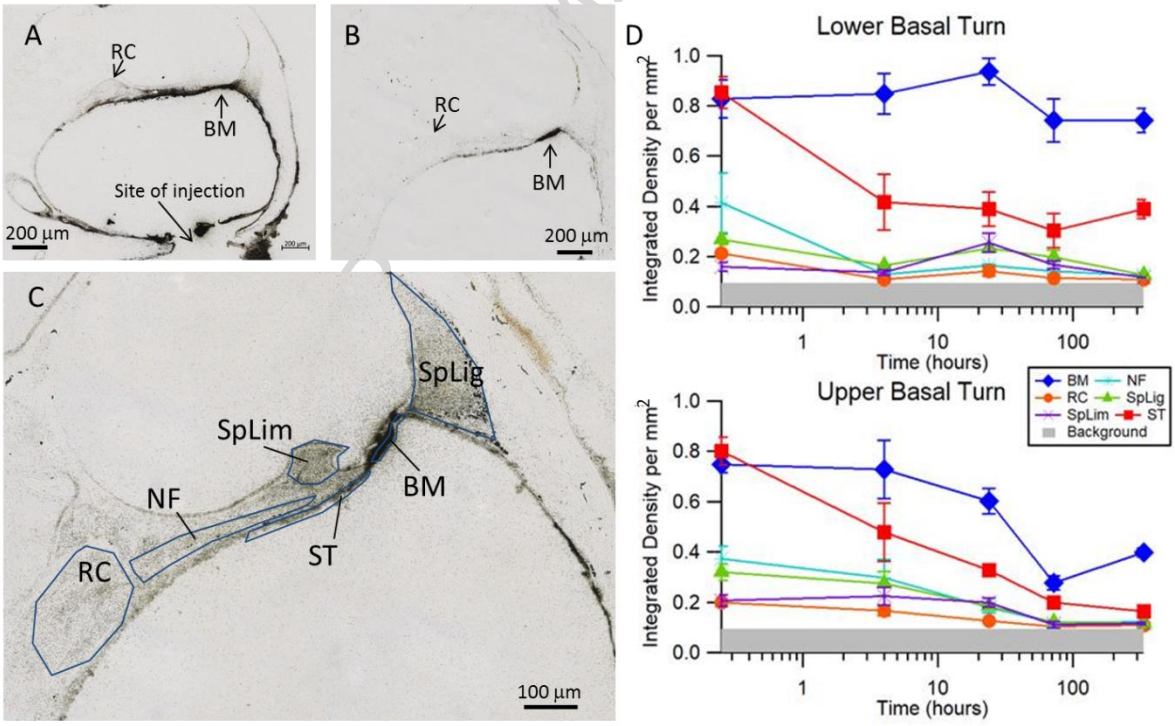

Figure 5: Non-uniform tissue distribution and tissue clearance Unstained autoradiographs of the lower basal turns from a representative cochlear section from the 15 minute group **(A)** and the 2 week group **(B)**. **(C)** Image from the 1 day group showing the regions of interest used for

measurement of radiolabel integrated density. RC=Rosenthal's canal, NF=peripheral nerve fibres, SpLim-spiral limbus, ST=cells lining scala tympani, BM=basilar membrane, SpLig=spiral ligament. **(D)** Normalised integrated density per mm^2 of radiolabel in each area of interest in the lower basal turn and the upper basal turn for each time point. Data is the average of the normalised data from the 1 week, 2 week and 4 week exposure data sets and error bars indicate the standard error of the mean (from n=3 exposure sets each containing 2-3 cochleae per group).

Concentration does not affect distribution pattern

Cochleae from Figure 1 were used to examine the effect of the dosing concentration on the tissue penetration and distribution pattern. These cochleae were injected with 5 µL of the Ntf3 dosing solution at three concentrations (0.9 µg/mL, 75 µg/mL or 750 µg/mL; the latter two dosing solutions consisted of a 1:100 or 1:1000 ratio of labelled to unlabelled Ntf3. i.e. 0.75 µg/mL ^{125}I Ntf3 mixed with 75 µg/mL or 750 µg/mL unlabelled Ntf3) injected at a rate of 0.2 µL/min. Cochleae were extracted after 4 hours for whole-cochlea gamma measurements followed by sectioning and autoradiography. For all concentrations, the ^{125}I Ntf3 signal was detected in all turns with a similar pattern of distribution (Figure 6). Integrated density was calculated across the entire cochlear cross section. The integrated density from the tissue-bound ^{125}I Ntf3 signal in the 750 µg/mL group was 6 times greater than the 75 µg/mL and 1378 times greater than the 0.9 µg/mL group (with adjustments made for the ratio of labelled to unlabelled Ntf3) (Figure 6).

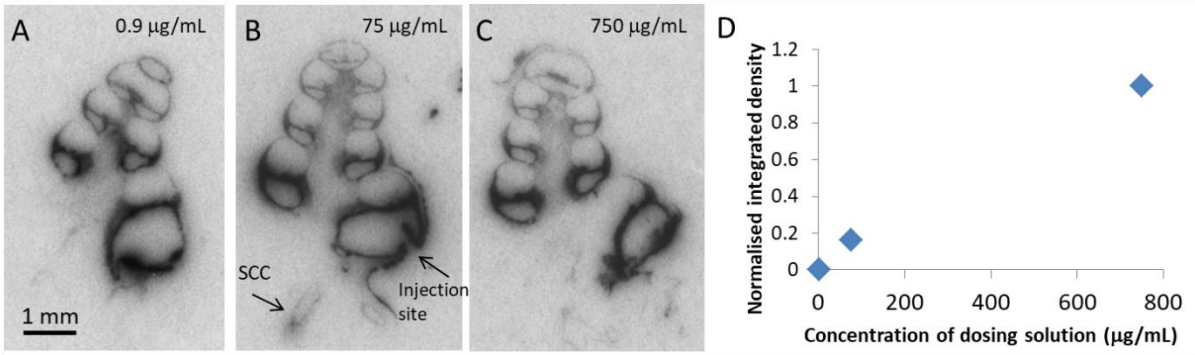

Figure 6: Effect of concentration on tissue distribution. Film autoradiographs from cochleae injected with 0.9 µg/mL **(A)**, 75 µg/mL **(B)** or 750 µg/mL Ntf3 **(C)** (with ^{125}I Ntf3 component being 0.75 µg/mL in **B** and **C**). **(D)** Average integrated density measurement from cochlear sections (adjusted for the ratio of labelled to unlabelled Ntf3) at the three dosing concentrations. SCC: semi-circular canal

Detection in ipsilateral semi-circular canals and minor spread to contralateral cochlea

Distribution of ^{125}I Ntf3 was also examined in non-cochlear tissues including blood, brain and semi-circular canals. ^{125}I Ntf3 was detected by film and slide-based emulsion autoradiography in the semi-circular canals in the sampled tissue from eight out of ten animals (Figure 7). The ^{125}I Ntf3 signal was detected in the perilymphatic spaces of the semi-circular canals, which contains loose connective tissue, but not in the cells lining the endolymphatic space. There were no overt post-surgical signs of disturbed balance in any of the guinea pigs although balance was not implicitly tested. No signal was detected in blood samples (gamma measurements), or in the midbrain, pons or medulla of any animal (emulsion coated slide autoradiography). ^{125}I signal in non-injected contralateral cochleae was only detected by film autoradiography with no specific ^{125}I Ntf3 signal detected by emulsion coated slide autoradiography or in control extra-cochlear tissue in the cochlear apex (Figure 7).

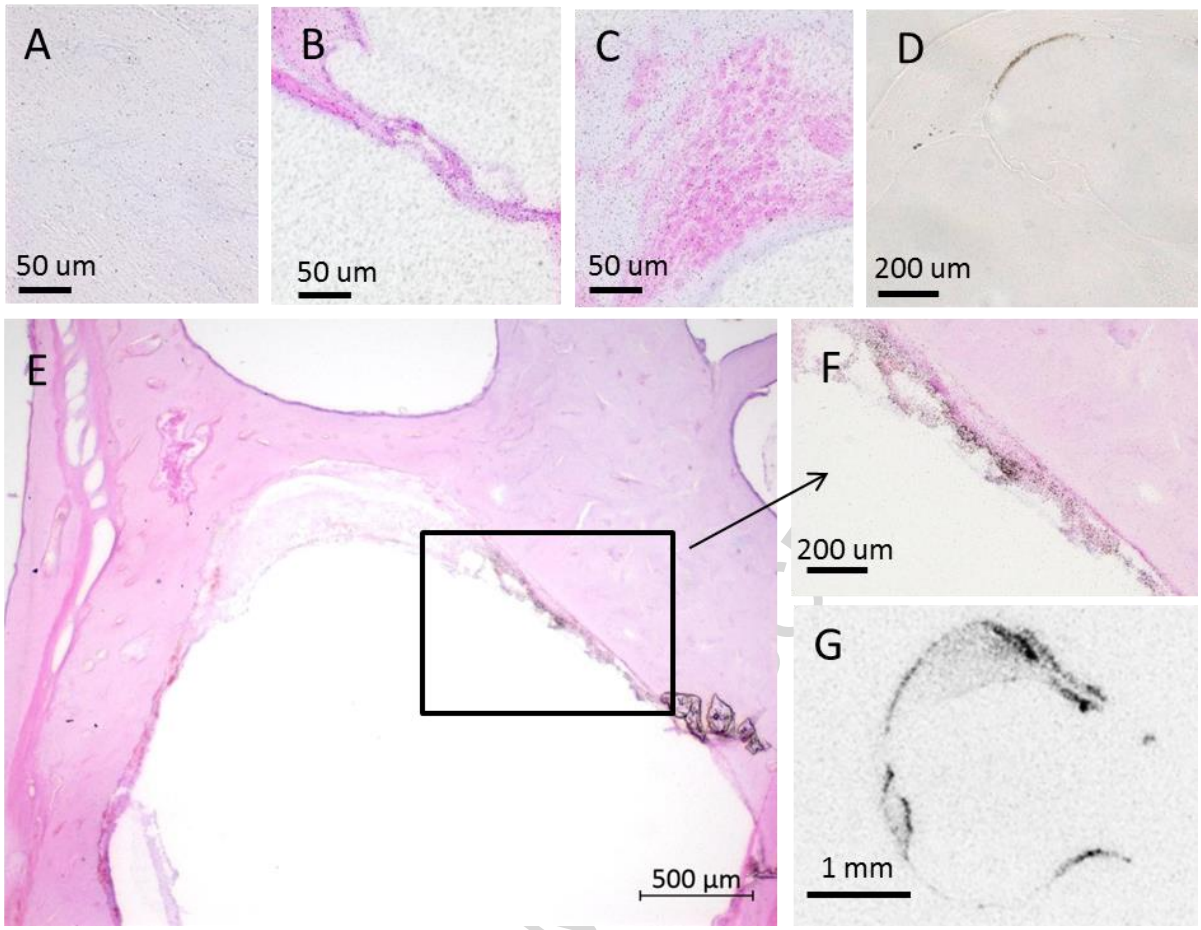

Figure 7: Contralateral and distant spread of ^{125}I Ntf3. H&E stained autoradiographs of cochlear sections showing **(A)** extra-cochlear control tissue on the injected side taken from the lateral bone near the cochlear apex, **(B)** contralateral side organ of Corti region and **(C)** contralateral side Rosenthal's canal region, each showing background signal only. **(D)** Contralateral side from the 3 day group film autoradiography showing only the pigmented cells of the stria vascularis and occasional pigmented cells lining the scala tympani. **(E)** H&E stained image of a semi-circular canal. The relatively small perilymphatic space contains connective tissue with ^{125}I Ntf3 signal. The square region is enlarged in **(F)**. **(G)** A similar region from a film autoradiograph.

Discussion

Endogenous growth factors are established, successful therapeutic modalities in drug discovery and the neurotrophin family of proteins has been extensively studied as potential therapies.

Encouragingly, a recombinant human nerve growth factor (Cenegermin) has recently been approved by European Medicines Agency and the Food and Drug Administration for ocular indications. There are now a number of drug delivery strategies under development for clinically translatable neurotrophin delivery to the cochlea but a systematic study of Ntf3 disposition in the inner ear has been lacking. The ability to directly compare the efficiency of drug delivery techniques would be a valuable tool to help determine the most effective strategy for different indications or aetiologies of hearing loss. In this study we used a radiolabelling technique to examine Ntf3 retention, clearance and distribution in a model of direct administration into a sealed cochlea, a potential clinical route for drug delivery as an adjunct to cochlear implantation.

Measurement of pharmacokinetics using a radiolabelled protein

Pharmacokinetic studies in the cochlea are typically performed by fluid sampling but the resolution of this technique is relatively low with the entire cochlear perilymphatic fluid from the scala tympani being approximately 4 µL and the sample volumes being 1 µL[22, 27]. Furthermore, fluid sampling informs about drug concentrations in the cochlear fluid, but gives no indication of whether the drugs reach their target tissue and in what proportion. Clearance and distribution measurements using radiolabelled Ntf3 shown here have the benefits of being measured in an intact cochlea without any compromise to the cochlea structure prior to fixation. The method enables the visualisation of drug levels in extracellular spaces of the cochlear tissue and potentially intracellular uptake in cells and the study of clearance and distribution over time via autoradiography.

Dosing volume and drug retention

The study showed a clear inverse relationship between the dosing volume and the percentage of drug retained in the cochlea at early time points. In terms of the total dose retained in the cochlea at the 4 hour time point, the data suggests there is little benefit gained in injecting larger volumes. In the guinea pig, a dosing volume of 5 µL represents 50% of the entire perilymphatic volume[28]. With

the cochlea encased in the bony otic capsule there is no capacity for volume expansion so during injection of a drug sample, fluid must be concurrently eliminated from the cochlea. This most likely occurs via the cochlear aqueduct: an anatomical connection between the perilymph of the cochlea and the subarachnoid space of the brain. The cochlear aqueduct enters into the scala tympani of the cochlea adjacent to the round window membrane and contains loosely packed connective tissue with a lumen of variable patency[29, 30]. Cerebrospinal fluid (CSF) normally flows into the cochlea through the cochlear aqueduct but following injection of a drug the flow may be reversed to facilitate release of pressure[31]. Inter-animal differences in the patency of the cochlear aqueduct or volume of the perilymph compartment may account for the variability observed for the 5 µL injection volume group. The variability of the 5 µL data at the 4 hour time point makes it difficult to interpret whether there was an effect of rate on the retention of the dosing material, but it is reasonable to assume that if the rate is slow enough to not significantly affect the volume of the cochlea at any given time (e.g. in the order of nL/min) retention rates may be considerably higher.

In addition to the cochlear aqueduct, other possible elimination routes have been proposed including the internal auditory canal and the apical part of the otic capsule[32-34]. The film and emulsion autoradiographs show evidence of ^{125}I Ntf3 signal within the internal auditory canal supporting this as an elimination route where perilymph may flow towards the brain following increased perilymphatic pressure[35]. While ^{125}I Ntf3 was not detected in the brain in this or a previous study[24], it is possible that it was below the detection limit. The overall thickness of the otic capsule is variable in the guinea pig cochlea. The extent of tissue perfusion from the perilymph into the lateral bone was limited to a distance of less than 100 µm in the base. However, at the apex where the otic capsule is thinnest the ^{125}I Ntf3 signal was observed via film autoradiography to penetrate the entire thickness of the otic capsule supporting this as another possible elimination route. External ^{125}I Ntf3 signal was detected near the cochlear apex but also near the injection site in the basal turn. It is therefore unclear whether this is evidence of elimination through the thin otic capsule at the cochlear apex or part of the dosing solution that has seeped between the cochlea and the

membrane surrounding the cochlea during injection. As a final elimination pathway, the perilymphatic space is lined with mesothelial cells which are porous and have a possible role in removal of debris [35]. Small regions of denser ^{125}I Ntf3 signal were apparent in some of the mesothelial cells suggestive of accumulation or uptake in these cells. There was also evidence of accumulation of ^{125}I Ntf3 signal around blood vessels and is another possible clearance route.

Distribution

Autoradiography images clearly demonstrate passage of ^{125}I Ntf3 from the perilymph into the cochlear tissue with evidence of perfusion through the osseous spiral lamina, spiral ligament and spiral limbus. The osseous spiral lamina contains micropores called canaliculi perforantes that allow extensive fluid communication between the scala tympani and Rosenthal's canal [36]. The prevalence of ^{125}I Ntf3 signal in the spiral ligament and osseous spiral lamina and the rapid clearance from these structures demonstrate unimpeded passage of perilymph through the osseous spiral lamina which is highly porous in experimental animals as well as in humans [36, 37]. This data supports other studies that hypothesise a direct communication route from the scala tympani to the scala vestibuli (i.e. through tissue rather than via the spiralling perilymphatic fluid space) [23, 38-43]. Simulations and drug distribution studies confirm that the rate of appearance of markers or drugs in the cochlear apex following basal injection or round window application exceed the expected rate based on the flow of the perilymph alone, predicted to be 19-21 nL/min [23, 38-43]. The endothelial cells lining the scala media are surrounded by tight junctions and do not permit perilymph to flow into the endolymph. The presence of ^{125}I Ntf3 signal in these endothelial cells suggests accumulation of Ntf3 in these cells due to the tight junction barrier.

We observed high amounts of ^{125}I Ntf3 in the basilar membrane region. Similar findings were reported following horse radish peroxidase injection into the middle ear cavity and in our previous studies on radiolabelled Ntf3 distribution in the cochlea [24, 25, 42]. The basilar membrane contains layers of collagen, beneath which is a tympanic covering layer: cells which have incomplete tight

junction network forming a tightly interwoven labyrinth-like environment[35, 44]. This may explain the slow clearance of ^{125}I Ntf3 and other molecules in this region. The cortilymph of the organ of Corti is believed to be continuous with the perilymph via gaps beneath the Deiter cells[35]. ^{125}I Ntf3 was observed in the reticula lamina but not within or between supporting cells and hair cells in the organ of Corti at any time point.

Basal to apical concentration gradient

Concentration gradients of drugs in cochlear tissues have not been previously measured to our knowledge. We observed a gradient in the amount of radiolabelled Ntf3 that was present in the tissue of the cochlea following direct injection into the cochlear fluids. After 4 hours, the density of the ^{125}I signal was 5 times greater in the basal turn compared to the apical turn. This is in agreement with a concentration gradient measured by fluid sampling following delivery of fluorescent-labelled dextran to the cochlea via a modified electrode array (implanted into the basal turn of the cochlea) connected to a pump that continually delivered the marker over 2 hours, 24 hours or 7 days. There was 5-6 times more labelled dextran near the base compared to the apex following two hours of continuous delivery and even after 7 days of constant slow-rate infusion of the drug into the cochlea, suggesting a higher than expected elimination rate and progressive loss of fluid from the cochlea in the higher turns of the cochlea[27]. The study also confirmed that the concentration of the drug in the cochlear fluids was highly dependent on whether the injection site was fully sealed as seepage of the drug out of the cochlear opening reduces the intracochlear concentration. At the level of neuronal survival from neurotrophin therapy after chemically induced hearing loss in guinea pigs, sustained intracochlear delivery of high concentrations of neurotrophins via a mini-osmotic pump is effective across most turns, but declining towards the apex[14, 20, 45].

Dosing concentration

Increasing the concentration of the dosing material increased the percent retained in the cochlea at the 4 hour time point. Therefore, a higher dosing concentration translates to higher prolonged doses in the cochlea via increased retention in addition to the more concentrated starting material. The overall tissue uptake increased with dose without affecting the distribution pattern. It is currently unknown how these results translate into the percent of bioavailable Ntf3 for the target tissue, the spiral ganglion neurons. ^{125}I Ntf3 was observed in the tissue surrounding spiral ganglion neurons. There was no obvious accumulation of ^{125}I Ntf3 within spiral ganglion neuron cell bodies, the target tissue in which the neurotrophin Trk receptors are expressed[46, 47]. Instead, ^{125}I Ntf3 signal appeared to be between the spiral ganglion neurons where glial cells such as Schwann cells are present. Cochlear Schwann cells, as well as cells within the osseous spiral lamina, have been reported to express the low affinity receptor p75[48].

The concentration of neurotrophic factors that is effective in preserving the spiral ganglion neuron population after profound hearing loss is in the order of 30-100 µg/mL when administered via a slow release device such as a mini-osmotic pump (approximately 6-20 µg of Ntf3 and BDNF over 28 days). This dose also stimulates the regrowth of peripheral fibres towards the organ of Corti[14, 18, 49]. In a study that directly compared Ntf3 that was continuously delivered to the cochlea at 10 µg/mL and 1 µg/mL, the 10 µg/mL concentration was more effective at protecting outer hair cells and reducing ABR threshold shift after noise exposure compared to 1 µg/mL Ntf3 [50]. However, a comprehensive dose comparison study has not been performed. Furthermore, factors such as protein precipitation at higher concentrations and protein degradation over time complicate the determination of therapeutic doses in studies using chronic neurotrophin delivery.

Limitations

The technique described effectively shows the passage of Ntf3 through the cochlea and the effects of concentration, volume and time, but it does not easily distinguish between non-specific binding and specific uptake by cells with Trk receptors. The technique is limited to proteins that can be radiolabelled without affecting bioactivity which we have shown for Ntf3 previously [24]. Long-term assessment of clearance in also limited by the half-life of the radiolabel which is 60 days for ^{125}I as well as the long exposure times required for film or emulsion-based autoradiography (up to 4 weeks). There is the overriding presumption that the technique of fixation by perfusion does not affect the distribution of the radiolabelled protein. To this end we performed a comparative protocol where the cochleae were snap frozen at 4 hours following injection and found no difference in the percent retention of the radiolabelled Ntf3.

Conclusions

This study describes a tool to study retention, clearance and distribution of drugs at the cellular level. Radiolabelling of Ntf3 along with gamma measurement and autoradiographic imaging methods permit the measurement of the total dose remaining in the cochlea at given time points in addition to the proportion of drug in the target tissue itself. We combined the high sensitivity of analysis by film autoradiography with the high cellular resolution achieved via emulsion-based autoradiography. This technique will enable comparison of the effectiveness of different drug delivery methods to deliver therapeutic agents to the target tissue. This study highlighted that direct, intracochlear delivery of Ntf3 into a sealed cochlea resulted in much of the administered dose distributing through the bony cochlear tissue and binding non-specifically to tissues and membranes. A small proportion was detected in Rosenthal's canal which contains the target tissue of neurotrophins, the spiral ganglion neurons.

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