Aus der Klinik und Poliklinik für Hals-, Nasen-, Ohrenheilkunde,

Der Ludwig-Maximilians Universität München

Direktor: Prof. Dr. med. Martin Canis

Zur Rolle der Mikrozirkulation in der Pathophysiologie der Cochlea

Kumulative Habilitationsschrift

Zur Erlangung der Venia Legendi

In der experimentellen HNO-Heilkunde

vorgelegt von

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aus Bonn

Dicebat Bernardus Carnotensis nos esse quasi nanos gigantum umeris insidentes, ut possimus plura eis et remotiora videre, non utique proprii visus acumine, aut eminentia corporis, sed quia in altum subvehimur et extollimur magnitudine gigantea

Johannes von Salisbury, Metalogicon

Meinen Eltern

Einführung

Das Hören gehört zu den wichtigsten Sinnen, die dem Menschen zur Verfügung stehen – nicht nur werden über das Hören nach dem Sehen die meisten Informationen über die Umwelt aufgenommen; der Großteil der zwischenmenschlichen Kommunikation läuft über die Sprache ab. Immanuel Kant formulierte daher einmal den Satz „*Nicht sehen können trennt von den Dingen, nicht hören können trennt von den Menschen“*. Die Diagnostik und Therapie sowie das Verständnis von Hörstörungen haben daher in der modernen Hals-, Nasen-, Ohrenheilkunde eine hervorgehobene Stellung.

Die Epidemiologie von Hörstörungen aller Art haben in den letzten Dekaden merklich zugenommen.[1,2] Ein Grund hierfür kann in der zunehmenden Lebenserwartung der Menschen gesehen werden, welche mit einer höheren Prävalenz für die altersbedingte Schwerhörigkeit einhergeht.[3] in einer Erhebung von 2017 waren in Süddeutschland etwa 16.2% aller erwachsenen von einem -größtenteils milden – Hörverlust betroffen;[2] wobei erwartet wird, dass diese Prävalenzzahlen etwa alle 5 Jahre um 1% steigen werden.[2] Im Jahre 2010 waren Hörstörüungen für kumuliert 19,9 Millionen Jahre mit Behinderung (years lived with disability) verantwortlich, womit auf die Hörstörungen insgesamt 2,6% aller Gesundheitsstörungen ausmacht.[4] Damit stellt die Schwerhörigkeit auch gesellschaftlich wie Volkswirtschaftlich ein erhebliches Problem dar.

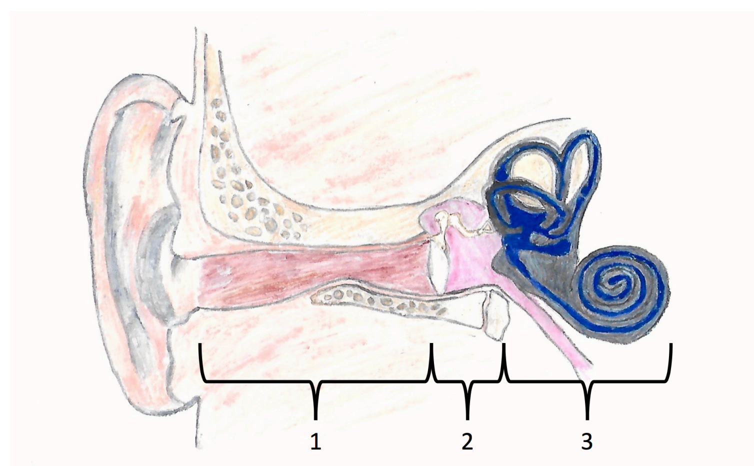

Abbildung 1 – Schematische Darstellung des Ohres mit äußerem Ohr und Gehörgang (1), Mittelohr (2) und Innenohr (3)

Klinisch werden die Schwerhörigkeiten vor allem in zwei relevante Kategorien unterteilt: die Schallleitungs- und die Schallempfindungs-schwerhörigkeit. Bei der Schallleitungsschwerhörigkeit ist der Schallleitungsapparat, also der Gehörgang, das Trommelfell oder die Gehörknöchelchen (Hammer, Amboß, Steigbügel) beeinträchtigt (Abbildung 1, Nr. 1 + 2). Bei der Schallempfindungsschwerhörigkeit sind die Haarzellen

im Innenohr (Abbildung 1, Nr. 3), welche die mechanische Energie von Schallwellen in elektrische Impulse umwandeln, geschädigt.

Schallleitungsschwerhörigkeiten haben häufig eine entzündliche Genese; so zählen die chronische Otitis media, welche in einen mesotympanalen und epitympanalen Typen unterteilt wird, zu den häufigsten Ursachen. Die genaue Ätiologie dieser Erkrankungen ist nicht in der Gänze geklärt, jedoch scheint eine Belüftungsstörung des Mittelohres mit einem chronischen Unterdruck eine zentrale Rolle zu spielen.[5]

Bei den Schallempfindungsschwerhörigkeiten handelt es sich in den meisten Fällen um den Endpunkt einer Reihe von Erkrankungen des Mittel- und des Innenohres, in dessen Verlauf es zu einer irreversiblen Schädigung der Haarzellen kommt. Zu den häufigsten Ursachen zählen Hörsturz[6], akute oder chronische Lärmexposition[7], Strahlenexposition[8], Entzündungen[9] und otoxische Medikamente[10,11].

In der Vergangenheit wurde wiederholt postuliert, dass Beeinträchtigungen der cochleären Mikrozirkulation und ein subsequenter Abfall des Sauerstoffpartialdruckes[12] in der Cochlea sowie ein Zusammenbruch der strialen Blut-Cochlea Barriere[13] zu dauerhaften Schädigungen der Haarzellen führen. In einem Teil der vorliegenden Arbeit sollen daher die Strukturen identifiziert werden, über welche Beeinträchtigungen der cochleären Mikrozirkulation vermittelt werden sowie Strategien evaluiert werden, um die reversiblen Veränderungen der cochleären Mikrozirkultion aufzuheben.

Eine weitere Erkrankung, bei der es im Verlauf zu einer erheblichen Schallempfidnungsschwerhörigkeit kommt, ist der M. Ménière. Zwar stehen initial vestibuläre Symptome im Vordergrund, im weiteren Verlauf der Erkrankung kommt es jedoch regelhaft zu einer zunehmenden Schallempfindungsschwerhörigkeit, welche Schlussendlich häufig zu einer funktionellen Ertaubung auf dem betroffenen Ohr führt.[14]

Die Therapie des M. Ménière ist vielfältig, zielt jedoch klinisch in erster Linie darauf ab die Anzahl der Schwindelanfälle zu reduzieren. Hierzu wird häufig Gentamicin oder Dexamethason intratympanal angewendet[15] oder orale Diuretika verschrieben. Eine weitere Möglichkeit besteht in der oralen Dauertherapie mit Betahistin, einem

Histaminanalogon, von dem angenommen wird, es reduziert die Anzahl der Schwindelattacken.[16,17]

Einer der angenommen Hauptwirkmechanismen von Betahistin die dosisabhängige Steigerung der cochleären Mikrozirkulation, welche den dem M. Ménière zu Grunde liegenden Endolymphhydrops reduzieren soll.[18] In der vorliegenden Arbeit soll daher der Wirkmechanismus von Betahistin auf die cochleäre Mikrozirkulation evaluiert werden.

Zielsetzung und Fragestellung der Arbeit

Mit der vorliegenden Habiliationsleistung soll ein Beitrag zum Verständnis der Pharmakologie und Pathophysiologie der cochleären Mikrozirkulation geleistet werden. In den dieser kamulativen Habiliation zu Grunde liegenden einzelnen Veröffentlichungen wurden daher folgende Fragestellungen bearbeitet:

- Welche Rolle spielt die cochleäre Mikrozirkulation in den Pathologien des Innenohres?
    - Tragen Perizyten zu entzündlichen Änderungen in der cochleären Mikrozirkulation bei?
    - Können entzündliche Abfälle der cochleären Mikrozirkulation durch das Sphingosid-1-Phosphat Analogon FTY-720 aufgehoben werden?
- Wie wirkt das Pharmakon Betahistin auf die cochleäre Mikrozirkulation?
    - Haben die Metaboliten Aminoethylpyridin, Hydroxyethylpyiridin und Pyridylessigsäure einen eigenen Effekt auf die cochleäre Mikrozirkulation?
    - Über welche Rezeptoren wird die Wirkung von Betahistin auf die cochleäre Mikrozirkulation vermittelt?
    - Gibt es spezifische cochleäre Strukturen, die die Wirkung von Betahistin auf die cochleäre Mikrozirkulation vermitteln?

Material und Methoden

In allen dieser Habilitationsschrift zu Grunde liegenden Originalarbeiten wurde das gleiche Tiermodell verwendet. Dementsprechend wurden alle relevanten Versuche unter den Aktenzeichen 55.2-1-54-2532-131-10 (Regierung von Oberbayern, München) bzw. 33.9-42502-04-12/0889 und 33.9–42502–04–14/1427 (Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg) bei den entsprechenden zuständigen Behörden registriert und genehmigt.

Versuchstiere

In allen hier angeführten Originalarbeiten wurden weibliche Dunkin-Hartley Meerschweinchen verwendet. Alle Versuche wurden als Akutversuch durchgeführt; nach der Beendigung des experimentellen Protokolls wurden die Versuchstiere durch eine letale intravenöse Gabe von Xylazin/Ketamin euthanasiert. Anschließend wurde noch eine zervikale Luxation durchgeführt.

Chirurgisches Vorgehen

Das chirurgische Vorgehen war bei allen Tieren nahezu identisch und ist bereits mehrfach beschrieben worden.[19,20] Die Narkose wurde durch regelmäßige i.m. Injektionen von Ketamin und Xylazin aufrechterhalten. Um Flüssigkeit und Kontrastmittel zu verabreichen wurde ein i.v.-Zugang in die linke V. jugularis gelegt. Anschließend wurde das ipsilaterale Ohr entfernt, das Felsenbein eröffnet und die Cochlea freigelegt. Durch vorsichtiges Abheben eines Knochenfenstern mit einem Skalpell wurde die Stria vascularis freigelegt, so dass diese Mikroskopiert werden konnte. Im Fall der 2-Photonen-Mikroskopie wurde prinzipiell gleichartig vorgegangen, jedoch wurde der Zugang durch das Felsenbein erweitert und auf eine Knochenfensterung verzichtet.

Intravitalmikroskopie

Den Meerschweinchen wurde über den zuvor angelegten i.v.-Katheter Fluoresceinisothiocyanatdextran verabreicht. Anschließend wurde mit einem Leica M205FA Stereofluoreszenzmikroskop die Stria vascularis dargestellt und Videoaufnahmen hiervon gemacht. Diese wurden digital Abgespeichert und mit dem

Programm CapImage ausgewertet, so dass nach vorheriger Eichung der intravaskuläre Blutfluss in Pikolitern/Sekunde quantifizert werden konnte.

Um Perizyten der Stria vascularis zu visualisieren, wurde für etwa 30 Minuten Diaminofluorescein-2-Diacetat topisch verabreicht und die Bulla anschließend ausgepült.

Versuchsgruppen

In der Ersten Teil der Versuche wurde den Versuchstieren zunächst Betahistin oder äquimolare Mengen von dessen Metaboliten, Aminoethylpyridin, Hydroxyethylpyridin und Pyridylessigsäure verabreicht. Außerdem wurde die Trägersubstanz NaCl 0,9% als Placebo verabreicht. Die Mikrozirkulation wurde 3 Minuten vor und 15 Minuten nach Injektion quantifiziert. Die Gruppengröße betrug 6 Meerschweinchen.

Im folgenden Versuch wurde Betahistin gemeinsam mit einem Placebo sowie einem H_1-Agonisten und -Antagonisten, einem H_3-Agonisten und Antagonisten sowie einem α_2-Antagonisten intravenös verabreicht und die Mikrozirkulation wurde 3 Minuten vor und 15 Minuten nach Injektion gemessen. Die Gruppengröße Betrug 6 Meerschweinchen.

Im letzten Versuch, welcher sich mit der Wirkung von Betahistin auseinandersetzt wurde wurden in 12 Meerschweinchen insgesamt 154 Perizyten angefärbt. Anschließend erhielt eine Hälfte Betahistin und die andere Hälfte ein Placebo. Intravasaler Blutfluß sowie Durchmesser an Orten von Perizytensomata sowie an flussabwärts gelegenen Kontrollstellen ohne Perizytensomata wurde vor und 7,5 Minuten nach Betahistingabe gemessen. Außerdem wurden in zwei Meerschweinchen der Durchmesser der präkapillären Arteriolen mittels 2-Photonen-Mikroskopie vor und 7,5 Minuten nach Betahistingabe quantifiziert.

In dem zweiten Teil der Versuche wurde zunächst in 12 Meerschweinchen insgesamt 199 Perizyten angefärbt. Zunächst wurde die intravasale Fließgeschwindigkeit sowie der Gefäßdurchmesser an Orten von Perizytensomata sowie an stromabwärts gelegenen Kontrollen ohne Perizytensomata gemessen. Anschließend wurde für 20 Minuten Tumornekrosefaktor (TNF) in 8 Tieren oder ein Placebo in vier Tieren appliziert und die genannten Parameter erneut gemessen. Hiernach wurde in der Gruppe die ein Placebo erhalten hatte erneut ein Placebo verabreicht, und die Gruppe, die TNF erhalten hatte wurde erneut in zwei Gruppen geteilt, von der eine ein Placebo erhielt und die andere den TNF-Antagonisten Etanercept. Schlussendlich wurden alle Parameter erneut gemessen und die Tiere euthanasiert.

In der letzten Veröffentlichung wurde bei den Versuchstieren zunächst Basalparameter erhoben, um anschließend TNF oder ein Placebo topisch zu applizieren. Anschließend wurde erneut die Mikrozirkulation gemessen und entweder FTY-720 oder ein Placebo appliziert und die Mikrozirkulation hernach ein letztes mal gemessen. Schlussendlich wurden die Versuchstiere euthanasiert.

Statistik

Die Statistische Analyse erfolgte mit Project R (Version 3.2.5 for Windows, The R Project for Statistical Computing, http://www.r- project.org/). Die Wahrscheinlichkeit für einen Fehler erster Art von $\alpha < 0.05$ wurde als statistisch signifikant angesehen.

Ergebnisse

Der Effekt von Betahistin und dessen Metaboliten auf die cochleäre Mikrozirkulation.

In der Gruppe, welche Betahistin erhielt, zeigte sich in den Minuten 7-13 sowie in den Minuten 15-18 eine Signifikant erhöhte cochleäre Mikrozirkulation im Vergleich zum Placebo; der höchste Wert wurde in Minute 8 mit 144% vom Ausgangswert erreicht (Abbildung 2, links)

In den Gruppen welche äquimolare Mengen Aminoethylpyridin und Hydroxyethylpyridin gegeben wurde, zeigten sich ebenfalls signifikant erhöhte Werte für die cochleäre Mikrozirkulation in den Minuten 6-18 (Aminoethylpyridin) bzw. 7-11 (Hydroxyethylpyridin). Der Scheitelwert wurde in Minute 8 mit einem Anstieg auf 153% von den Basalwerten (Aminoethylpyridin, Abbildung 2, rechts) bzw. in Minute 7 mit einem Anstieg auf 127% von den Basalwerten (Hydroxyethylpyridin) beobachtet.

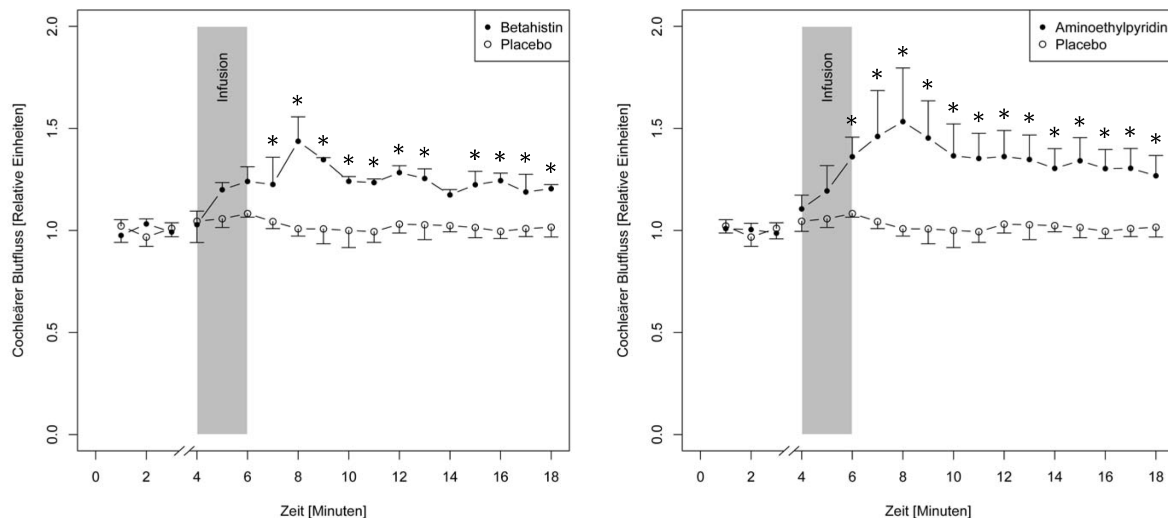

*Abbildung 2 – Der Effekt von Betahistin (links) und Aminoethylpyridin (rechts) auf die Cochleäre Mikrozirkulation. * = p<0.05 (two way ANOVA)*

Rezeptoren, über welche die Wirkung von Betahistin auf die cochleäre Mikrozirkulation vermittelt wird.

Die Gruppe, welche Betahistin gemeinsam mit Diphenhydramin, einem H_1-Antagonisten erhielt, zeigte sich keine statistisch relevante Veränderung im Vergleich zu der Gruppe, in welcher Betahistin gemeinsam mit einem Placebo verabreicht wurde. (Abbildung 3, links) In der gruppe, in der der H_3-Antagonist Thioperamid mit Betahistin verabreicht wurde, kam im weiteren Verlauf es zu keiner relevanten Veränderung der Ausgangswerte und zu einem statistisch signifikanten Unterschied zwischen dieser Gruppe und jener, welcher Betahistin mit einem Placebo erhielt. (Abbildung 3, rechts)

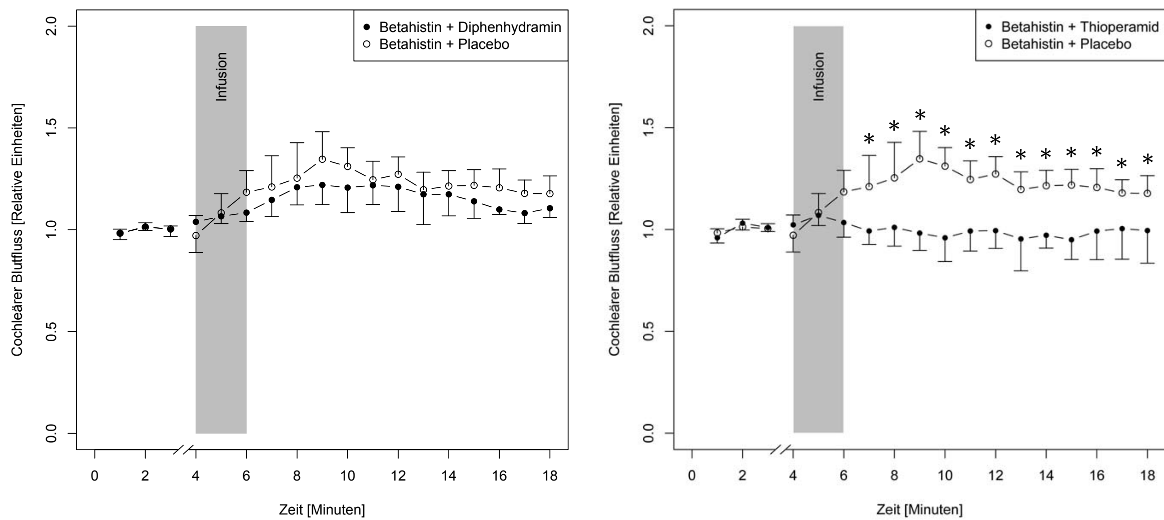

*Abbildung 3 – Der Effekt von Betahistin gemeinsam mit dem H_1-Antagonisten Diphenhydramin (links) und gemeinsam mit dem H_3-Antagonisten Thioperamid (rechts) auf die cochleäre Mikrozirkulation. Als Placebokontrolle wurde Betahistin gemeinsam mit einem Placebo verabreicht. * = $p<0.05$ (two way ANOVA)*

Betahistin +	Wirkmechanismus	Ergebnis
Diphenhydramin	H_1-Antagonist	Keine Veränderung d. Betahistinwirkung
α-Methylhistamin	H_3-Agonist	Abnahme von CBF
Thioperamid	H_3-Antagonist	Aufhebung der Betahistinwirkung
Proxyfan	Funktionell selektiver H_3-Agonist	Aufhebung der Betahistinwirkung
Yohimbin	α_2-Antagonist	Keine Veränderung d. Betahistinwirkung

Tabelle 1 – Zusammenfassung der Ergebnisse der verschiedenen Rezeptoragonisten und -antagonisten, welche mit Betahistin kombiniert i.v. verabreicht wurden

Der Effekt von Betahistin auf die präkapillären Arteriolen und die kapillären Perizyten der Stria vascularis

Die präkapillären Arteriolen zeigten nach 7,5 Minuten nach der intravenösen Gabe von Betahistin eine erhebliche Dilatation, welche sich signifikant vom Ausgangswert unterschied (Abbildung 4, rechts). Hingegeben gab es bei keinem der untersuchten Perizyten der Stria vascularis 7,5 Minuten nach intravenöse Gabe von Betahistin eine relevante Diltation im Vergleich zu einer Placebokontrolle oder den flußabwärts gelegenen Kontrollmessungen ohne Perizytensomata (Abbildung 4, links).

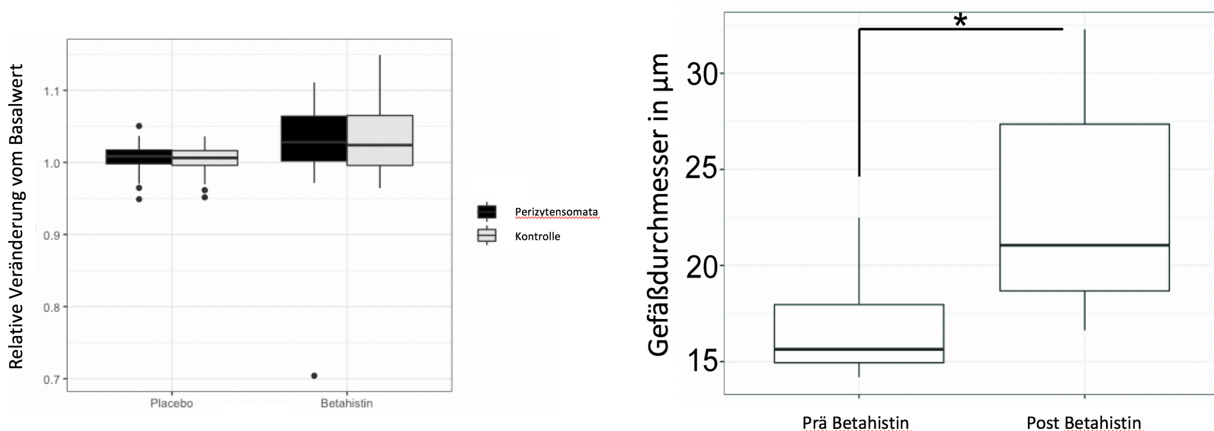

*Abbildung 4 – Der Effekt von Betahistin auf die Perizyten der Stria vascularis (links) und auf die präkapillären Arteriolen (rechts) vor und 7,5 Minuten nach Infusion. * = p<0.05, Mann-Whitney Test*

Der Effekt von topischem TNF auf die cochleäre Mikrozirkulation und striale Perizyten

Nach topischer Applikation von TNF kam es zu einer signifikanten Verminderung des Kapillardurchmessers sowohl an Orten von Perizytensoma (3.6±4.2%) und an den stromabwärts gelegenen Kontrollpunkten ohne Perizytensoma (2.3±2.9%), verglichen mit Placebo (0.2±2.0% Perizytensoma bzw. 0.4±2.5% Kontrolle). Es bestand aber auch ein statistisch signifikanter Unterschied zwischen den Orten von Perizytensoma und den stromabwärts gelegenen Kontrollpunkten nach Applikation von TNF (Abbildung 5, links). Nach Anschließender Gabe von Placebo oder Etanercept kam es zwischen allen Gruppen sowohl an Orten von Perizytensomata (0±2.7% Placebo/Placebo, 0.4±2.4% TNF/Placebo, 3.3±5.5% TNF/Etanercept) als auch an den Kontrollpunkten (0.2±2.7% Placebo/Placebo, 0.4±2.5% TNF/Placebo, 1.8±5.5% TNF/Etanercept) zu signifikanten Unterschieden

zwischen den einzelnen Gruppen. Weiterhin bestanden signifikante Unterschiede zwischen Perizytensoma und Kontrollpunkten in der TNF/Etanercept Gruppe.

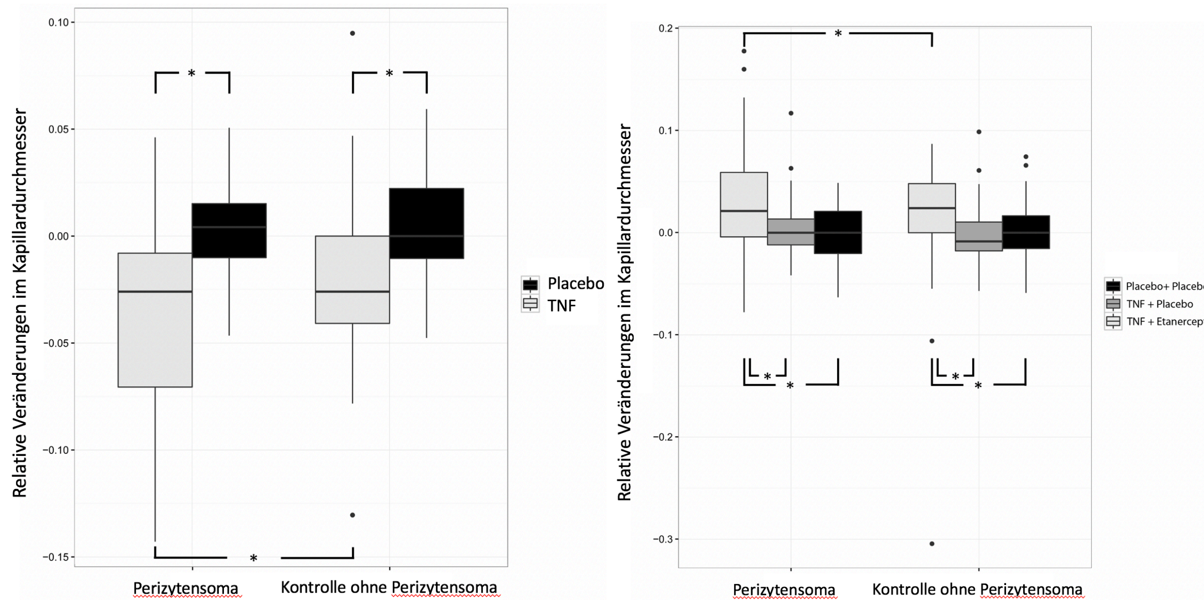

*Abbildung 5 – Der Effekt von TNF oder Placebo auf den Kapillardurchmesser an Orten von Perizytensoma oder stromabwärts gelegenen Kontrollpunkten ohne Perizytensoma (links) sowie der Effekt von anschließender Applkiktion von Placebo oder Etanercept (rechts). * p<0.05*

Die Wirkung von FTY-720 auf die cochleäre Mikrozirkulation nach Applikation von TNF

Initiale topische Applikation von TNF führte zu einem Abfall der cochleären Mikrozirkulation auf 81±7% vom Ausgangswert; dieser Unterschied ist im Vergleich zu der Applikation von Placebo (101±6% vom Ausgangswert) signifikant. Die Anschließende Applikation von Placebo führte zu keiner relevanten Veränderung der Mikrozirkulation; der Wert verweilte bei 81±10% vom Ausgangswert. Anschließende Applikation von FTY-720 hingegen führte zu einem Anstieg der cochleären Mikrozirkulation auf 94±7% vom Ausgangswert. Dieser Wert unterschied sich statistisch signfikant von der Gruppe, in welcher TNF und anschließend ein Placebo appliziert wurde, hingegen nicht signifikant von der Gruppe, in der ausschließlich Placebo appliziert wurde.

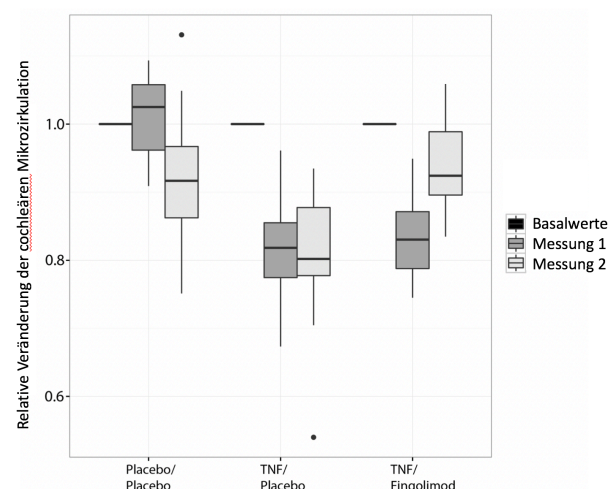

Abbildung 6 - Die Wirkung von TNF und FTY-720 auf die cochleäre Mikrozirkulation

Diskussion

Die am meisten angewendete Methode zur Quantifizierung war in der Vergangenheit stets die Laser-Doppler-Flussmessung.[21–23] Der sicherlich wichtigste Vorteil dieser Methode ist die einfache Handhabung – eine entsprechende Messsonde wird auf die präparierte Cochlea aufgesetzt und gibt automatisch relative Veränderungen im cochleären Blutfluss an. Jedoch werden bei dieser Methode auch Gefäße der Mucosa des Mittelohres, weiterer knöcherner Mittelohrstrukturen sowie der A. spiralis modioli miterfasst, welche nicht zur Mikrozirkulation der Cochlea zählen.[24]
In den dieser Arbeit zu Grunde liegenden Originalarbeiten[25–27] wurde stets die Intravitalmikroskopie zur Quantifizierung der cochleären Mikrozirkulation verwendet. Da hierbei die Stria vascularis chirurgisch freigelegt und unzweifelhaft identizifiert wird, sind Messungen mit dieser Methode deutlich spezifischer für die Stria vascularis und weniger Störanfällig durch andere Gefäße.[19,28,29] Daher erlauben die mit dieser Methode generierten Daten validere Beobachtungen von Veränderungen der Mikrozirkulation und dadurch Rückschlüsse auf mögliche Beeinträchtigungen im Metabolismus des Corti-Organs.

Die Tatsache, dass nicht nur Betahistin, sondern auch äquimolare Mengen der Metaboliten Aminoethylpyridin und Hydroxyethylpyridin den cochleären Blutfluss im Vergleich zu einem Placebo signifikant steigern zu vermögen, hat mehrere Implikationen. Einerseits erscheinen die Metaboliten von Betahistin als möglicher Weg, wie Betahistin eine langfristige Wirkung in der Ménière'schen Erkrankung entfalten könnten, da der Wirkstoff selber nur kurzfristig oder garnicht nach oraler ingestion im Plasma nachweisbar ist.[30,31]
Weiterhin besteht die Möglichkeit, dass Betahistin selbst eigentlich nur ein Prodrug ist, welches im Rahmen der Abbauprozesse zu dem eigentlich aktiven Metaboliten, Aminoethylpyridin, abgebaut wird. Nicht auch zuletzt erscheint Aminoethylpyridin aber in Anbetracht der mindestens zu Betahistin gleichwertigen Wirkung auf die cochleäre Mikrozirkulation als alternatives Therapeutikum zu Betahistin-Hydrochlorid; um diesen Punkt besser zu adressieren, sind jedoch noch eine Reihe von weiteren Untersuchungen notwendig.

Die Beobachtung dass die cochleäre Mikrozirkulation nach intravenöser Gabe von Betahistin ansteigt; dieser Effekt aber durch eine Blockade des H_3-Rezeptors, nicht jedoch des H_1-Rezeptors aufgehoben werden kann, legt die Vermutung nahe, dass die Wirkung von Betahistin auf die cochleäre Mikrozirkulation über den inversen Agonismus Betahistins am H_3-Rezeptor[32] vermittelt wird.
Relevant ist hierbei vor allem die Tatsache, dass Betahistin nicht als einfacher Antagonist am H3-Rezeptor wirkt, sondern als inverser Agonist; der Unterschied besteht darin, dass ein reiner (kompetitiver) Antagonist lediglich die Bindung eines Liganden an einen Rezeptor verhindert wohingegen der inverse Antagonist den Rezeptor bindet, und dessen Spontanaktivität herabsetzt.[33] Diese Erkenntnis hat insbesondere klinische Relevanz; eine gleichzeitige Verabreichung von Medikamenten mit einem reinen H_3-Agonismus sollte nach dem aktuellen Kenntnisstand vermieden werden; dies gilt jedoch nicht für die in der Allergietherapie eingesetzten H_1-Antagonisten[34]. Im Gegenteil könnte ein spezifischerer Wirkstoff mit einem selektiveren Profil für den H3-Rezeptor ein besseres Nebenwirkungsspektrum aufweisen, da die typischerweise unter Betahistintherapie berichteten Nebenwirkungen klassische H1-Rezeptor vermittelte Reaktionen sind.[35,36]

Im Rahmen einer weiteren Serie von Experimenten wurde die Beobachtung gemacht, dass Betahistin nach intravenöser Applikationen zwar keinen Effekt auf die mittels DAF2-DA angefärbten kapillären Perizyten bzw. auf den an den Orten von Perizytensomata gemessenen Kapillardurchmesser hat; es zeigt sich jedoch, dass es nach intravenöser Gabe zu einer relevanten Vasodilatation von präkapillären Arteriolen kam.
Die zentrale Erkenntnis dieser Beobachtung ist, dass der cochleären Mehrdurchblutung nach intravenöser Gabe von Betahistin mit hoher Wahrscheinlichkeit ein spezifischer Effekt für die Cochlea zu Grunde liegt. Sollte es nämlich zu einer generalisierten Vasodiltation in den Versuchstieren kommen, wäre ein Abfall der cochleären Mikrozirkulation zu erwarten.[37] Zu dem gemessenen Zeitpunkt war bei der verabreichten Menge Betahistin jedoch in vorangegangenen Studien jedoch stets der Höchstwert für die Steigerung der cochleären Mikrozirkulation gemessen worden.[18,38,39]
Auch die Tatsache, dass die präkapillären Arteriolen von einigen[40,41] (jedoch nicht von allen[42]) Autoren aufgrund ihrer Morphe zu den Perizyten gezählt werden spricht für einen spezifischen Effekt von Betahistin auf die cochleäre Mikrozirkulation.

Die Tatsache, dass es nach topischer Applikation von Tumornekrosefaktor zu einem Abfall der Mikrozirkulation kommt, ist bereits mehrfach in der Literatur vorbeschrieben worden.[43,44] Mechanistisch wurde bisher postuliert, dass diese Beobachtung durch einen prokontrstriktiven Zustand der präkapillären Arteriolen zu erklären ist.[45] Eine weitere Struktur, die die Wirkung von Tumornekrosefaktor vermitteln könnte, sind die kapillären Perizyten der Cochlea. Ähnliche Beobachtungen sind bereits im Zentralnervensystem beschrieben worden.[40,41]

Die Tatsache, dass sich der Gefäßdurchmesser an Orten von Perizytensomata nach Exposition von Tumornekrosefaktor signifikant einerseits von der Kontrollgruppe, aber auch von stromabwärts gelegenen Kontrollpunkten ohne Perizytensomata unterscheidet, legt einen spezifischen Effekt von Perizytzen auf den Gefäßdurchmesser der Kapillaren nahe. Auch die Tatsache, dass sich dieser Effekt nach Neutralisation durch Etanercept, ein Fusionsprotein aus dem TNF-Rezeptor und dem F_c-Teil eines IgG-Antikörpers, aufheben lässt, untermauert diese Hypothese.

Passend hierzu wurden in mehreren Perizytensubpopulationen kontraktile Proteine nachgewiesen,[46] was ebenfalls für eine aktive Regulation des cochleären Blutflusses auf kapillarer Ebene durch Perizyten suggeriert. Zwar wurden kontraktile Subpopulationen der cochleären Perizyten bereits zuvor beschrieben,[47] jedoch ist die Tatsache, dass dies auch auf einen (patho-)physiologischen Entzündungsstimulus hin passiert sowie die Reversibilität zuvor nicht beschrieben worden.

Bedenkt man, dass die cochleäre Mikrozirkulation eine wichtige Rolle für eine Reihe von Innenohrerkrankungen spielt, wie beispielsweise Hörsturz[45,48], Lärmtrauma[49] oder M. Ménière[18], erscheint eine ähnliche Pathophysiologie der cochleären Perizyten und der zerebralen Perizyten bei Ischämischen Ereignissen wahrscheinlich. So wurde für die Perizyten des ZNS beschrieben, dass diese sich im Rahmen eines ischämischen Ereignisses in eine Art *Rigor mortis* begeben und auch nach einer erfolgreichen Rekanalisation zu einer persistierenden Minderdurchblutung betroffener Areale führt.[40]

Die Beobachtung, dass FTY-720 die Wirkungen von Tumornekrosefaktor auf die cochleäre Mikrozirkulation aufheben kann, ist vermutlich durch den Agonismus am Sphingosin-1-Phosphat-Rezeptor erklärt werden: Der pharmakologisch ähnliche Wirkstoff JTE-013 zeigt ähnliche Wirkungen auf die cochleäre Mikrozirkulation nach

Tumornekrosefaktor-exposition.[43] Ein weiterer potenzieller Wirkmechanismus ist der kompetitive Antagonismus am Cannabinoid CB_1-Rezeptor: für FTY-720 wurde genau ein solcher Wirkmechanismus nachgewiesen,[50] wobei in einem Tiermodell des Darmes gezeigt werden konnte, dass der durch LPS verursachte Abfall der Mikrozirkulation durch einen Antagonismus am CB_1-Rezeptor aufgehoben werden kann. Interessanterweise wurde auch berichtet, dass CB1- und CB2-Rezeptoren in vitro (in inneren Haarzellen)[51] und in vivo (in stria vascularis und inneren Haarzellen)[52] habituell exprimiert und nach apoptotischem Stress durch Cisplatin hochreguliert werden, so dass dieser Signalweg als valides alternativziel erscheint.
Da ähnliche Effekt auf die Mikrozirkulation für Etanercept im Tiermodell nachgewiesen wurden[43,44] und dieses sich im Tiermodell auch günstig bei Lärmtrauma[49] sowie anekdotisch auch im Menschen begünstigend bei Hörsturz[45] auswirkt, erscheint FTY-720 für diese Pathologien als mögliches Therapeutikum.

Zusammenfassung

Insgesamt konnte im Rahmen der vorliegenden Habilitationsschrift gezeigt werden, dass Betahistin einen spezifischen Effekt auf die cochleäre Mikrozirkulation, vermittelt durch präkapilläre Arteriolen, ausübt. Auch konnte gezeigt werden, dass diese Wirkung durch den inversen Agonismus von Betahistin am Histamin H3-Rezeptor vermittelt wird, was neben einem besseren Verständnis der Pharmakologie von Betahistin auch klinische Implikationen hat.

Auch konnte gezeigt werden, dass nicht nur Betahistin selbst, sondern auch dessen Abbauprodukte, Aminoethylpyridin und Hydroxyethylpyridin einen Effekt auf die cochleäre Mikrozirkulation aufweisen – so dass diese vermutlich auch zum klinsichen Effekt von Betahistin bei der Ménière'schen Erkrankung beitragen.

Die Tatsache, dass FTY-720 die Wirkung von Tumornekrosefaktor auf die cochleäre Mikrozirkulation aufheben kann, bedeutet dass dieses Pharmakon als Potenzielles Therapeutikum bei Hörsturz und Lärmtrauma zu betrachten ist. Weiterhin werfen diese Bobachtungen ein neues Licht auf den Cannabinoid-Rezeptor in der Cochlea, der weitere Untersuchungen verdient.

Nicht zuletzt konnte gezeigt werden, dass sich cochleäre Perizyten auf Exposition gegen Tumornekrosefaktor kontrahieren und relaxieren können. Dies zeigt einerseits, dass die cochleären Perizyten an der Regulation der Mikrozirkulation aktiv teilnehmen. Andererseits erscheint aber auch eine gewichtige Rolle in der Pathophysiologie von allen Entitäten, die mit Beeinträchtigungen der Mikrozirkulation einhergehen, wie Lärmtrauma, toxischer Innenohrschädigung oder Hörsturz, und ähnlich wie im Zentralnervensystem schon beschrieben, als sehr wahrscheinlich.

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Faksimiledrucke der zu Grunde liegenden Publikationen

1. Bertlich M.*, Ihler F.*, Sharaf K., Weiss B., Strupp M., Canis M., *Betahistine metabolites aminoethylpyridine, and hydroxyethylpyridine increase cochlear blood flow in guinea pigs in vivo*. Int J Audiol 2014;53(10):753-759 *contributed equally
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3. Bertlich M., Ihler F., Freytag S., Weiss B., Strupp M., Canis M., *Cochlear pericytes are capable of reversably decreasing capillary diameter in vivo after tumor necrosis factor exposition*, Otol Neurotol, 2017 Dec;38(10):e545-e550.
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4. Bertlich M., Ihler F., Freytag S., Weiss B., Jakob M., Strupp M., Canis M., *Fingolimod (FTY-720) is capable of reversing tumor necrosis factor induced decreases in cochlear blood flow*, Otol Neurotol, 2017 Sep;38(8):1213-1216
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Original Article

Betahistine metabolites, Aminoethylpyridine, and Hydroxyethylpyridine increase cochlear blood flow in guinea pigs *in vivo*

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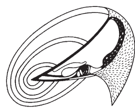
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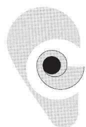
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Abstract

Objective: Betahistine is a histamine-like drug that is used in the treatment of Ménière's disease. It is commonly believed that betahistine increases cochlear blood flow and thus decreases the endolymphatic hydrops that is the cause of Ménière's. Despite common clinical use, there is little understanding of the kinetics or effects of its metabolites. This study investigated the effect of the betahistine metabolites aminoethylpyridine, hydroxyethylpyridine, and pyridylacetic acid on cochlear microcirculation. *Design:* Guinea pigs were randomly assigned to one of the groups: placebo, betahistine, or equimolar amounts of aminoethylpyridine, hydroxyethylpyridine, or pyridylacetic acid. Cochlear blood flow and mean arterial pressure were recorded for three minutes before and 15 minutes after treatment. *Study sample:* Thirty Dunkin-Hartley guinea pigs assigned to one of five groups with six guinea pigs per group. *Results:* Betahistine, aminoethylpyridine, and hydroxyethylpyridine caused a significant increase in cochlear blood flow in comparison to placebo. The effect seen under aminoethylpyridin was greatest. The group treated with pyridylacetic acid showed no significant effect on cochlear blood flow. *Conclusion:* Aminoethylpyridine and hydroxyethylpyridine are, like betahistine, able to increase cochlear blood flow significantly. The effect of aminoethylpyridine was greatest. Pyridylacetic acid had no effect on cochlear microcirculation.

Key Words: Betahistine; histamine; aminoethylpyridine; hydroxyethylpyridine; pyridylacetic acid; cochlear blood flow; Ménière's disease

When in 1861 Prospere Ménière described a condition that involved repeated attacks of one-sided hearing loss, tinnitus, and vertigo, he was the first to ascribe the aforementioned symptoms not to the central nervous system, but to the semicircular canals (Ménière, 1861). Despite the significant amount of time that has passed since the first description of Ménière's disease and notwithstanding the considerable prevalence in the population of up to 0.51% (Neuhauser, 2007), there is still extensive debate about its etiology, pathophysiology and treatment.

Nowadays, a wide range of treatments is administered, such as surgical approaches including surgery of the endolymphatic sac (Pullens et al, 2010), local application of gentamicin (Pullens & van Benthem, 2011) and more pharmacological therapeutic options like diuretics (Thirlwall & Kundu, 2006) or dietary restrictions (Boles et al, 1975).

The common approach in Europe is the oral administration of betahistine dihydrochloride, a structural derivative of histamine that shows inverse agonism on histamine H3-receptors and slight agonistic effects on histamine H1-receptors (Gbahou et al, 2010). Several studies suggest that betahistine might have a positive effect on the course of the disease (James & Burton, 2001; Strupp et al, 2011). Moreover, several clinical trials have found dose-dependent effects of betahistine on the frequency of attacks (Lezius et al, 2011). A sigmoid dose-response curve could also be reproduced in an animal model (Ihler et al, 2012). However, a solid and well-conducted, double-blind placebo-controlled prospective clinical study is still lacking.

So far it has been generally accepted that betahistine increases cochlear blood flow (Meyer et al, 1994; Laurikainen et al, 1998; Dziadziola et al, 1999; Lamm & Arnold, 2000). However, little is

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Abbreviations

ANOVA Analysis of variance
Arb Arbitrary

known about the pharmacokinetics of betahistine and its metabolites: A monoamino oxidase mediated strong first-pass effect has been suggested at the hepatic level. (Konzett et al, 1971; Sternson et al, 1974). It has been shown that the end product of betahistine metabolism is pyridylacetic acid, an inactive compound that can be found in both urine and plasma after oral betahistine ingestion (Chen et al, 2003; Val et al, 2010). Hypothesized degradation paths give rise to the metabolites aminoethylpyridine and hydroxyethylpyridine (Bowman et al, 1972; Sternson et al, 1974; Chen et al, 2003; Val et al, 2010). These metabolites have been shown to possess an affinity to histamine-receptors on their own (Fossati et al, 2001). However, the effects of these metabolites on cochlear blood flow have not been investigated so far. Moreover, even though identified as the end product of betahistine metabolism, neither has pyridylacetic acid been investigated for its potency to alter cochlear blood flow.

Therefore, the aim of the present study was to determine if either aminoethylpyridine, hydroxyethylpyridine, or the final metabolite of betahistine, pyridylacetic acid, may increase cochlear blood flow and to investigate their potency of action in comparison to betahistine.

Materials and Methods

Ethics statement

All experiments were performed according to state regulations for animal experimentation and were approved in April 2011 and December 2012 by the responsible authorities, the District Government of Upper Bavaria (Regierung von Oberbayern, Munich, Germany; animal license no.: 55.2-1-54-2532-131-10) and the animal protection services of Niedersachsen (LAVES, Oldenburg, Germany, animal license no.: 33.9-42502-04-12/0889).

Animals

Thirty Dunkin-Hartley Guinea Pigs purchased from Charles River Wiga Laboratories (Sulzfeld, Germany) and weighing 200–500 g were included in this study. Anesthesia was induced by inhalation of 1l O2/min, 0.5l N2H/min and 2.5 Vol% Isoflurane in a custom-made chamber. Thereafter, it was continued by an initial intraperitoneal injection of ketamine (50 mg/kg bw) and xylazine (5 mg/kg bw), and injections of half the aforementioned dosages every 30 minutes.

Surgical preparation

Surgical preparation and intravital microscopy were performed as described previously (Canis et al, 2010). In brief, a fiberoptic pressure transducer was placed in the right femoral artery for continuous blood pressure monitoring. A catheter was placed in the right jugular vein for intravenous application of fluids, plasma markers, and the agents that were to be tested. Following these initial preparations, the right auditory bulla was carefully opened and a rectangular window was incised in the second cochlear turn.

Measuring cochlear blood flow

After the initial preparation, blood flow of the capillaries of the stria vascularis was captured and quantified. This was achieved by an intravenous injection of fluorescein isothiocyanate labeled dextrane as a plasma marker in order to differentiate plasma from red blood cells. Selective observation of FITC-contrasted plasma was then performed using epi-illumination with a 100 W mercury lamp attached to a specific fluorescence filter block which was mounted on a modified microscope (Axiotech Vario, Carl Zeiss AG, Oberkochen, Germany). Filming was performed with an analogue video camera (C2400-08, Hamamatsu Photonics, Hamamatsu, Japan) and recorded on tape (Sony DVCAM DSV 45P, Tokyo, Japan). Velocity (μm/s) and diameter (μm) were measured using an offline image analysis system (CapImage, Dr. Zeintl Biomedical Engineering, Heidelberg, Germany) (Klyscz et al, 1997) and recorded in three different vessels every minute. A mean value was calculated afterwards.

In order to calculate cochlear blood flow, the formula established by Baker & Wayland in 1974: $q = (v/1.6) \times (d/2)^2 \times \pi$, was used (Baker & Wayland, 1974). In this formula, q represents the intravasal blood flow, v stands for the velocity of red blood cells inside the vessel and d is the vessel diameter.

Measurement of mean arterial pressure

Mean arterial pressure was measured using a Samba Fiber-Optic Pressure Measurement System by Samba Sensors AB (VästraFrölunda, Sweden) (Woldbaek et al, 2003). The tip of a fiber-optic catheter was inserted into the right femoral artery. During the experiment, the results were recorded with a Samba 201 Control Unit in millimeters of mercury (mmHg). Recording took place with a frequency of 40 Hz. The proprietary Samba 200 control software was used for later off-line analysis. To correct for differences between individual animals, changes in blood pressure are reported as arbitrary units.

Treatment protocol

Thirty animals were randomly assigned to one of five groups (betahistine, aminoethylpyridine, hydroxyethylpyridine, pyridylacetic acid, or placebo) and underwent identical microsurgery as described above. After an initial picture was obtained, baseline measurements were recorded for three minutes. Subsequently, betahistine, aminoethylpyridine, hydroxyethylpyridine, pyridylacetic acid, or placebo were administered over two minutes. From the beginning of the infusion, cochlear blood flow and arterial pressure were continuously monitored for a further 15 minutes.

Calculation of corresponding dosages for metabolites

A concentration of 0.1 mg betahistine per kg body weight was used, since it had been calculated to be equivalent to 48–160 mg of orally applied betahistine (Ihler et al, 2012), which is a dosage that is commonly applied in the clinic. Moreover, it has been shown that this dose is capable of significantly increasing cochlear blood flow without causing any adversive effects (Meyer et al, 1994; Ihler et al, 2012). Aminoethylpyridine and hydroxyethylpyridine were both applied in concentrations of 0.06 milligrams per kilogram body weight, whilst pyridylacetic acid was administered in a dose of 0.08 milligrams per kilogram

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body weight, representing equimolar amounts between the agents and betahistine.

Statistical analysis
Statistical analysis was carried out using SigmaPlot for Windows 12.0. The statistical test applied was a two-way repeated measures analysis of variance (ANOVA) in order to compare corresponding points in time between placebo and treatment groups (betahistine, aminoethylpyridine, and hydroxyethylpyridine respectively).

In order to correct for multiple testing for multiple groups and time-points, a Bonferroni t-test was performed. A p-value of $\alpha < 0.05$ was considered to be statistically significant.

Results

The effect of betahistine and its metabolites on cochlear microcirculation
Three of the four groups showed significantly increased levels of cochlear microperfusion in comparison to the placebo group. The group receiving betahistine showed an increase in cochlear perfusion to a peak value of 1.437 ± 0.120 arb units. Significant differences from placebo values were assessed from minutes 7 to 13 and 15 to 18 ($p < 0.05$; two-way repeated measures ANOVA/Bonferroni t-test). Cochlear perfusion in the betahistine group remained at a constant level after approximately 10 minutes. The average for minutes 10–18 was 1.227 arb units with a standard deviation of 0.034 arb units. In comparison to this, the average for the same period in the placebo group was 1.012 arb units with a standard deviation of 0.051 arb units (Figure 1, A).

The administration of aminoethylpyridine also led to a significant increase in cochlear perfusion in comparison to the placebo group ($p < 0.05$; two-way repeated measures ANOVA/Bonferroni t-test). At minute 11 a peak of 1.533 ± 0.263 arb units (range 2.015–1.306 arb units) could be observed, which exceeded the values achieved in the betahistine group. After an initial steep increase, cochlear blood flow remained constant at a level of approximately 1.3 arb units for the rest of the observation period. Overall, cochlear blood flow in the treatment group receiving aminoethylpyridine was significantly different from the values of the placebo group from minute 6 to minute 18 (Figure 2, A).

The group that received hydroxyethylpyridine showed significantly increased levels of cochlear perfusion in comparison to placebo ($p < 0.05$; two-way repeated measures ANOVA/Bonferroni t-test). After a moderate increase in cochlear perfusion at the beginning of metabolite infusion, a peak value of 1.268 ± 0.213 arb units was measured at minute 7. This peak level was below the maximum value in the betahistine group. The levels of cochlear perfusion remained steady at an average of 1.181 ± 0.159 arb units from minute 7 to minute 18. Overall, cochlear blood flow was significantly elevated in comparison to placebo from minute 7 to minute 11 (Figure 3, A).

The group that had been treated with pyridylacetic acid showed no significant changes in comparison to placebo (Figure 4, A).

The effect of betahistine and its metabolites on mean arterial pressure
Both betahistine and hydroxyethylpyridine groups showed statistically significant differences in mean arterial pressure compared to placebo.

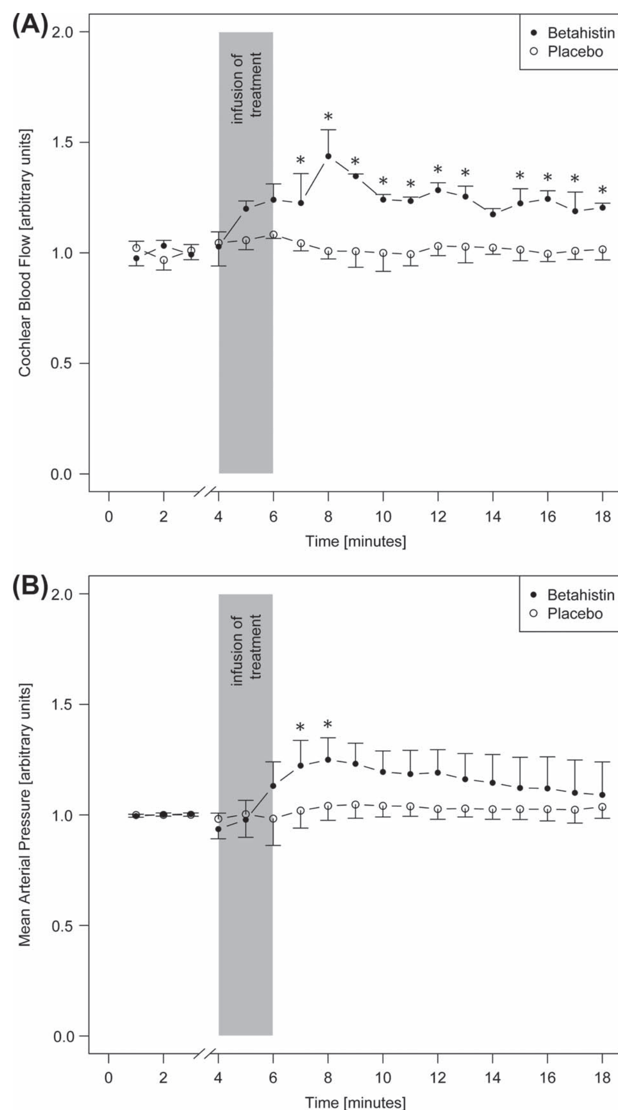

Figure 1. Effects over time before and after infusion of betahistine. (A) Cochlear blood flow; (B) Mean arterial pressure in arbitrary units; mean $\pm$ SE. *: $p < 0.05$ (two-way repeated measures ANOVA/ Bonferroni t-test).

The treatment group receiving betahistine displayed an initial slight drop in mean arterial pressure with the lowest value at the beginning of the infusion (mean value 0.936 arb units with a standard deviation of 0.044 arb units and ranging from 0.868–0.993 arb units). After this initial and short drop, the systemic blood pressure increased to levels significantly different from placebo at minutes 7 and 8 ($p < 0.001$, two-way repeated measures ANOVA/Bonferroni t-test), reaching a peak at minute 8 with 1.250 ± 0.99 arb units, and from there on kept constantly decreasing (Figure 1, B).

No statistical differences from placebo in terms of systemic blood pressure were noted in the treatment group with administration of aminoethylpyridine (Figure 2, B).

The group receiving hydroxyethylpyridine showed a constant increase in mean arterial pressure upon initial infusion, reaching a peak significantly different from placebo at minute 6 with 1.183 ± 0.124 arb units ($p < 0.001$, two-way repeated measures ANOVA/Bonferroni t-test). From then on, average values for

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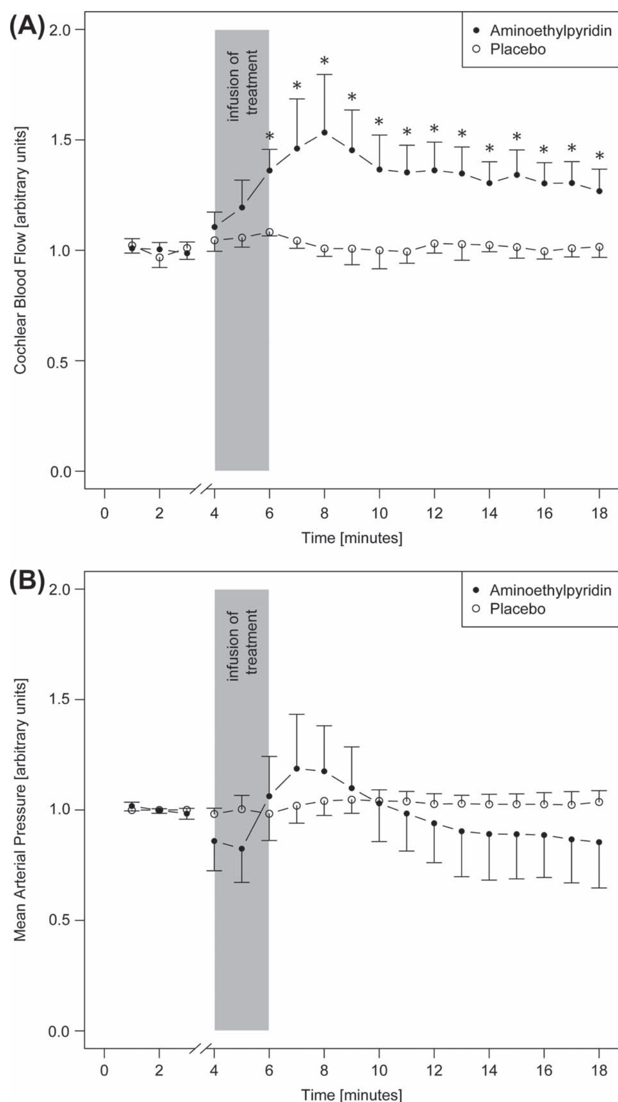

Figure 2. Effects over time before and after infusion of aminoethylpyridine. (A) cochlear blood flow; (B) mean arterial pressure in arbitrary units; mean ± SE. *: $p < 0.05$ (two-way repeated measures ANOVA/Bonferroni t-test).

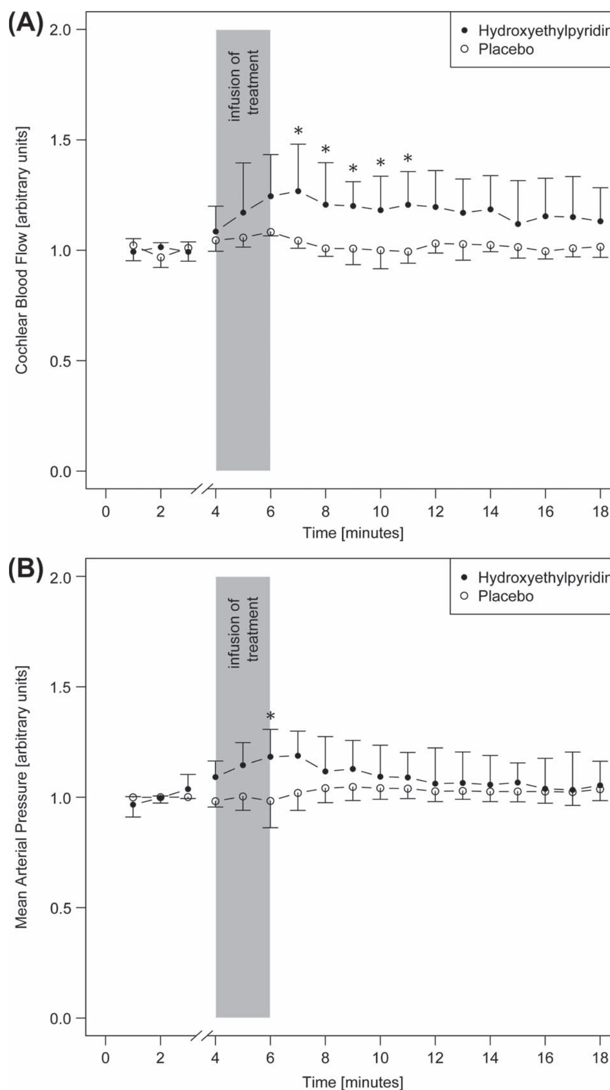

Figure 3. Effects over time before and after infusion of hydroxyethylpyridine. (A) cochlear blood flow; (B) mean arterial pressure in arbitrary units; mean ± SE. *: $p < 0.05$ (two-way repeated measures ANOVA/Bonferroni t-test).

systemic blood pressure gradually declined, eventually reaching the levels of the placebo group from minute 9 (Figure 3, B).

In terms of mean arterial pressure, the group that had received pyridylacetic acid had shown no significant changes in comparison to placebo (Figure 4, B).

Discussion

The major finding of this study on the effects of three metabolites of betahistine was that two of the three compounds caused a significant increase in cochlear blood flow. Aminoethylpyridine even exerted a major effect compared to betahistine. Hydroxyethylpyridine had an additional impact on systemic blood pressure in a dimension comparable to betahistine. Pyridylacetic acid, on the other hand, was unable to alter either systemic blood pressure or cochlear blood flow.

Betahistine acts as an agonist of histaminergic H1-receptors and as an inverse agonist of H3-receptors (Gbahou et al, 2010). It has been established in vitro that aminoethylpyridine has similar properties on the H3-receptor, whilst being a much weaker agonist of the H1-receptor (Fossati et al, 2001). Hydroxyethylpyridine is an even weaker agonist of the H1-receptor and an inverse agonist of the H3-receptor (Fossati et al, 2001). The receptor binding profile for pyridylacetic acid has not yet been investigated, however it has been repeatedly described as being practically non-existent (Botta et al, 2000; Fossati et al, 2001; Chen et al, 2003). As histamine is an omnipresent substance in the body, it is not surprising that it has considerable effects on the cardiovascular system. Histamine H1-receptors have been reported to cause negative inotropic effects on the heart as well, vasoconstriction in greater and vasodilation in smaller vessels, and a general drop in blood pressure (Sakai, 1980). In this respect, H3 receptors are similar: upon

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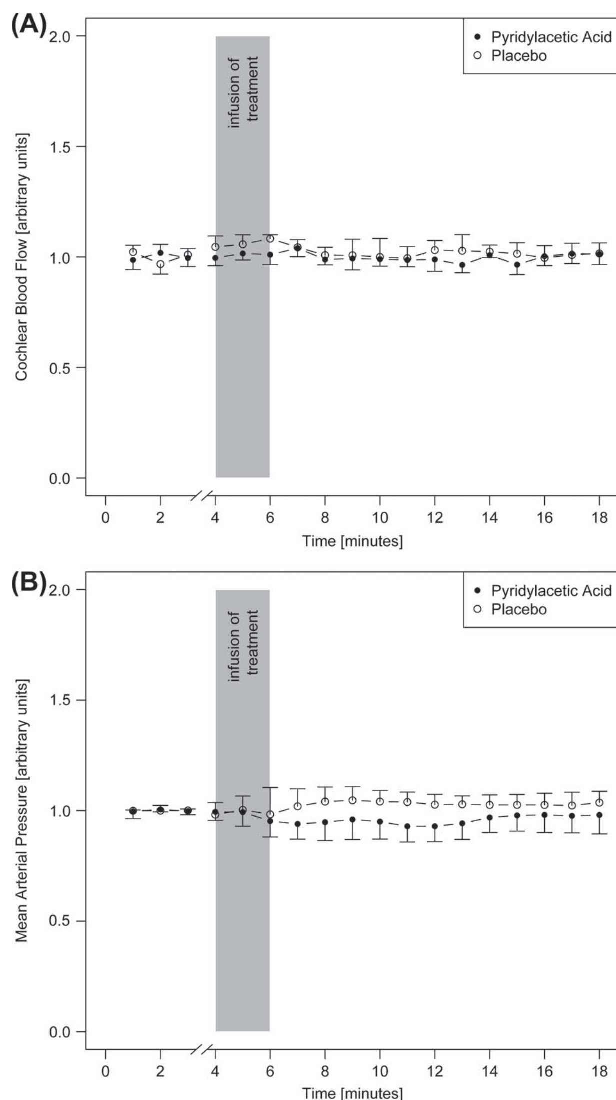

Figure 4. Effects over time before and after infusion of pyridylacetic acid. (A) cochlear blood flow; (B) mean arterial pressure in arbitrary units; mean ± SE. *: $p < 0.05$ (two-way repeated measures ANOVA/Bonferroni t-test).

activation they cause a drop in noradrenaline levels and a general decrease in blood pressure (Malinowska et al, 1998). This is commonly viewed as the most likely mode of action of betahistine in the inner ear. Histamine receptors, including that of the H3-subtype are present in various tissues of the inner ear (Dagli et al, 2008). It seems probable that these H3-receptors modulate local noradrenaline release for the arterioles of the stria vascularis, thus altering cochlear blood flow (Laurikainen et al, 1998). Fittingly, it has been described that α-methylhistamine, an H3-agonist with an effect directly opposite to that of betahistine at the H3-receptor, is capable of inducing a vasoconstriction in resistance vessels of prepared rat bowel (Sun et al, 2011).

There has not been much research on betahistine metabolites so far. However, it has been shown that aminoethylpyridine is able to decrease blood pressure in mongrel dogs (Konzett et al, 1971) during a two-minute infusion. This finding does not contradict the presented data: even though not statistically different from placebo, the guinea pigs receiving aminoethylpyridine infusions showed a drop in mean arterial pressure below basal values (Figure 2, B, minutes 4 and 5). However, apart from the initial drop in mean arterial pressure during aminoethylpyridine infusion, we could not find any data on either of the betahistine metabolites and their effect on mean arterial blood pressure.

The fact that hydroxyethylpyridine—unlike aminoethylpyridine—is able to generate a significant increase in mean arterial pressure, despite its aforementioned somewhat weaker potency at histamine H1- and H3-receptors (Fossati et al, 2001) implies that other receptors might be involved in the effects of betahistine and its metabolites on mean arterial pressure. Fittingly, it has been described that pretreatment of animals with idazoxane, an adrenergic α2- and imidazole I2-antagonist, is able to decrease the betahistine-typical changes in both mean arterial pressure and cochlear blood flow (Laurikainen et al, 1998). Hence adrenergic receptors might play a role in the mediation of the effect of betahistine and its metabolites.

Whilst we recorded mean arterial pressure in order to have a measure for the systemic effects of betahistine, it seems probable that the main effect of betahistine that is considered as beneficial takes place in the cochlear vascular network (Laurikainen et al, 1998, 2000). It has been shown that cochlear function and cochlear microcirculation are closely related (Ihler et al, 2012; Arpornchayanon et al, 2013). Moreover, the fact that the cochlea is a circulatorily privileged organ with a strong autoregulation of its blood flow (Kawakami et al, 1991; Brown & Nuttall, 1994) suggests that the effects observed are specific to the cochlea, thus rendering this the most likely mode of action of betahistine in Ménière's disease.

To this day, there are no studies that have investigated the effect of any of the metabolites on cochlear blood flow. Yet, there have been *in vitro* studies that have examined the ability of all three metabolites in comparison to betahistine to decrease the resting discharge rate of prepared frog's ampullar receptors (Botta et al, 2000, 2001). One major finding was in line with the results presented here. In both the aforementioned as well as in our experiment, aminoethylpyridine exerted effects that were very similar to that of betahistine on the dependent variable, whilst the effect of hydroxyethylpyridine and pyridylacetic acid was much smaller.

This study differs from earlier investigations into cochlear microcirculation and betahistine (Meyer et al, 1994; Laurikainen et al, 1998, 2000; Dziadziola et al, 1999) in the application of intravital microscopy for cochlear blood flow measurement. From the 1980s onwards, laser Doppler flowmetry has been the main method of measuring cochlear blood flow (Miller et al, 1983; Goodwin et al, 1984; Miller et al, 1984). An important limitation of laser Doppler flowmetry is that it is not selective for cochlear microcirculation because it does not exclusively assess stria vascularis vessels (LaRouere et al, 1989; Nakashima et al, 2001). However, with the assessment of cochlear perfusion, these are the relevant vessels responsible for cochlear metabolism. Instead, laser Doppler flowmetry will measure any vessel upon which it is placed and the blood flow in the vessel. Therefore, in our case, values generated with this method would include averages of the stria vascularis as well the spiral modiolar artery and vessels of the bony capsule of the cochlea (Nuttall, 1987; Canis et al, 2010). Although superior in specificity, one considerable limitation of this method is the time for which cochlear blood flow can be measured, since after approximately 20 minutes, the vessels observed show an increasing tendency to clot and thus no valid data can be measured anymore.

One has to acknowledge the effects exerted by aminoethylpyridine, which are at least similar to betahistine, (Botta et al, 2000,

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2001) or in the present study even greater than that observed under betahistine treatment. Hence, it is tempting to speculate whether the main therapeutic effect of medication with betahistine could be exerted by its metabolites. Could betahistine even act as a prodrug, and aminoethylpyridine or another metabolite as the main therapeutic agent? A prodrug is a partially or completely inactive precursor that is only fully converted into its active form at or near the site of action (Wu & Farrelly, 2007). In that respect, one should also acknowledge the fact that if one considers aminoethylpyridine as the major therapeutic agent, the greater the distance of the examined substance in the presumed metabolic pathway of betahistine, the smaller its effect. Moreover, the strong hepatic first-pass effect (Sternson et al, 1974) would further support this theory. However, the exact kinetics of betahistine and its metabolites in the plasma upon ingestion are unknown to this date.

Therefore, in further investigations the temporal kinetics of betahistine and its metabolites by route of delivery as well as their effect on the actual endolymphatic hydrops in an animal model should be examined (Kimura, 1967, 1982).

Conclusion

This study showed that the betahistine metabolites, aminoethylpyridine and hydroxyethylpyridine both exert an effect on systemic blood pressure and cochlear blood flow, whilst pyridylacetic acid had no effect whatsoever. It should encourage further research in this particular field such as chronic hydrops models and receptor studies to facilitate the investigation of betahistine metabolites and their role in the treatment of Ménière's disease.

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Histaminergic H_3-Heteroreceptors as a Potential Mediator of Betahistine-Induced Increase in Cochlear Blood Flow

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Key Words

Betahistine · H_3-receptors · H_1-receptors · α_2-receptors · Ménière's disease · Cochlea · Microcirculation · Fluorescence microscopy

Abstract

Objective: Betahistine is a histamine-like drug that is considered beneficial in Ménière's disease by increasing cochlear blood flow. Acting as an agonist at the histamine H_1-receptor and as an inverse agonist at the H_3-receptor, these receptors as well as the adrenergic α_2-receptor were investigated for betahistine effects on cochlear blood flow. ***Materials and Methods:*** A total of 54 Dunkin-Hartley guinea pigs were randomly assigned to one of nine groups treated with a selection of H_1-, H_3- or α_2-selective agonists and antagonists together with betahistine. Cochlear blood flow and mean arterial pressure were recorded for 3 min before and 15 min after infusion. ***Results:*** Blockage of the H_3- or α_2-receptors caused a suppression of betahistine-mediated typical changes in cochlear blood flow or blood pressure. Activation of H_3-receptors caused a drop in cochlear blood flow and blood pressure. H_1-receptors showed no involvement in betahistine-mediated changes of cochlear blood flow. ***Conclusion:*** Betahistine most likely affects cochlear blood flow through histaminergic H_3-heteroreceptors.

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Introduction

In 1861, Prosper Ménière was the first to ascribe a certain combination of tinnitus, one-sided hearing loss and an extreme feeling of vertigo not to the brain but to the inner ear [Ménière, 1861a, b]. Not much later, this triad of symptoms was being referred to as 'maladie de Ménière', Ménière's disease [Thorp and James, 2005].

The most common approach in Europe for the treatment of Ménière's disease is the continuous oral application of betahistine dihydrochloride. Betahistine has been used in the treatment of Ménière's disease for decades; hence clinical trials and meta-analyses of its efficacy are numerous. It is commonly accepted that repetitive daily doses of betahistine are capable of reducing the number and gravity of attacks during the course of the disease [Claes and Van de Heyning, 1997, 2000; James and Burton, 2001; James and Thorp, 2005].

However, to this day it is not clear how betahistine acts in Ménière's disease. It has been proposed that betahistine, through its histamine-like properties, might increase vascular permeability and thus decrease the endolymphatic hydrops that is the cause of Ménière's disease [Ber-

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lin et al., 2011]. Moreover, betahistine could aid in the central-nervous compensation that takes place after a patient has suffered from an attack [Redon et al., 2011]. Lastly, it has been shown that betahistine is capable of increasing cochlear blood flow in animal models and could therefore aid in the reduction of the endolymphatic hydrops [Dziadziola et al., 1999; Laurikainen et al., 2000; Ihler et al., 2012a]. So far, this has been viewed as the most likely mechanism of action in Ménière's disease [Strupp et al., 2011].

Betahistine is a structural analog of histamine that has been shown to act as a potent inverse agonist on histamine H_3-receptors [Gbahou et al., 2010] and as a weaker agonist on H_1-receptors [Fossati et al., 2001]. It is commonly accepted that betahistine has no effect whatsoever on histaminergic H_2-receptors [Curwain et al., 1972; Laurikainen et al., 1998; Fossati et al., 2001]. Moreover, there have been results that suggest that betahistine also affects another class of receptors, potentially of the adrenergic α-receptor subfamily [Dziadziola et al., 1999]. To this day, the receptors by which betahistine increases cochlear microcirculation have not been investigated systematically and have only been assessed in a scattered manner. A potential cause for this is the early approval of betahistine in the late 60s of the previous century, when a considerably lower pharmacological understanding of a drug was required for approval. Moreover, the exact mode of action of betahistine at the histaminergic H_3-receptor was only been discovered in 2010 [Gbahou et al., 2010]. To this day, the receptors investigated as mediators of betahistine effects have included histaminergic [Laurikainen et al., 1998; Dziadziola et al., 1999], cholinergic [Laurikainen et al., 1993], adrenergic [Laurikainen et al., 1998] and imidazole receptors [Laurikainen et al., 1998].

The aim of this study was to systematically evaluate the receptor or receptors that give rise to the increase in cochlear blood flow caused by betahistine.

Materials and Methods

Animals

A total of 54 healthy female Dunkin-Hartley guinea pigs (180–300 g) obtained from Charles River Laboratories (Sulzfeld, Germany) were included in the study. All experiments were performed according to German state regulations for animal experimentation and were approved by the responsible authorities, the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES, Oldenburg, Germany; animal license No. 33.11.42502-04-012/889).

The animals initially received buprenorphine 0.05 mg/kg body weight subcutaneously. Approximately 30 min after the initial application of buprenorphine, the animals were sedated using a mixture of ketamine (8.5 mg/kg body weight) and midazolam (0.75 mg/kg body weight). After the animals were fully sedated, anesthesia was continued throughout the experiments by the continuous inhalation of 3% isoflurane.

The preparative surgery in the experiments lasted on average about 90 min and the measurements 18 min. Following the experiments, the animals were euthanized.

Surgical Preparation and Intravital Imaging

Surgical preparation and intravital microscopy for measuring microcirculation parameters were performed as described elsewhere [Canis et al., 2010; Ihler et al., 2012b]. Utilizing microsurgery, a polyethylene catheter was placed in the left jugular vein for the application of fluids, agents and contrast material. A pressure transducer was placed in the right femoral artery. Finally, the right ear was removed and the underlying bulla carefully opened. A rectangular window of approximately 0.2 × 0.2 mm was carved into the exposed cochlea.

As previously described, intravital microscopy allowed direct examination and recording of stria vascularis vessels [Nuttall, 1987]. Utilizing FITC (fluorescein isothiocyanate)-labeled dextran (molecular weight 500,000; 0.2–0.4 ml of a 5% solution in 0.9% NaCl; Sigma, Deisenhofen, Germany) that had been injected intravenously as a plasma marker, it was possible to differentiate the intravasal erythrocytes from the FITC-dyed plasma. The images were obtained using illumination with a Leica EL6000 light source (Leica Microsystems, Wetzlar, Germany) linked to a Leica M205 FA stereomicroscope. The data generated was processed with the proprietary Leica Application Suite software and then saved on a digital hard drive for later off-line analysis. Velocity (micrometers per second) and diameter (micrometers) of stria vascularis vessels were measured after the surgical procedure with the image analyzation system Cap-Image (Dr. Zeintl Biomedical Engineering, Heidelberg, Germany) [Zeintl et al., 1989; Klyscz et al., 1997]. During analysis of the acquired data, three representative vessels for each animal were selected. For these vessels, three values for intravascular blood flow and three values for the respective diameter were obtained each minute. These values were then averaged for each minute and, utilizing the formula postulated by Baker and Wayland, they were used to calculate the intravascular blood flow for each minute. The formula was given as $q = (v/1.6) \times (d/2)^2 \times \pi$, where q represents the intravascular blood flow, v the intravascular velocity and d the vessel diameter [Baker and Wayland, 1974]. In order to correct for interindividual differences, cochlear blood flow was reported in arbitrary units (AU), thus reflecting the relative change from the initially obtained basal values.

The originally obtained basal values for intravascular blood flow ranged from 2 to 56 μl/s, depending on the animal and vessel examined. Potential reasons for this wide range of data sets include a possible impairment or injury of the vessels during the surgical preparation or drying out of the capillaries during fluorescence microscopy. Moreover, the fewer times a capillary had branched up before the point in which the measurements were taken, the greater the diameter and the larger the intravasal blood flow. To calculate relative change in cochlear blood flow, an average of the three basal values of each vessel was calculated. Any value recorded in this vessel was then divided by this average basal value. Finally, an average value for each minute was calculated from the relative change values for each vessel.

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Measurement of Mean Arterial Pressure

Mean arterial pressure was recorded using a Fiber-Optic Pressure Measurement System by Samba Sensors AB (Västra Frölunda, Sweden) [Woldbaek et al., 2003]. The fiber-optic catheter was inserted into the right femoral artery. For the duration of the experiments, the results were automatically recorded with a Samba 201 Control Unit, with a rate of 40 measurements per second. The ensuite Samba 200 control software was used for later analysis of the acquired data. The basal data sets for mean arterial pressure ranged from 14 to 79 mm Hg. Potential reasons for this data set include early circulatory failure caused by prolonged surgical preparation and interindividually different reactions to the anesthesia caused by variations in age or weight of the animals.

To correct for differences between individual animals, changes in blood pressure are reported as AU, reflecting the relative change. AUs were calculated by dividing each value obtained for mean arterial pressure by an average of the three basal values obtained for each individual.

Calculation of Normalized Cochlear Blood Flow

Normalized cochlear blood flow [Baldwin et al., 1992; Ohlsen et al., 1992] was calculated by dividing the obtained arbitrary values for cochlear blood flow by the arbitrary values obtained for the mean arterial pressure, allowing us to report a relative change in cochlear blood flow without units, corrected for potential systemic influences.

Treatment Protocol

The 54 animals were randomly assigned to one of nine groups (betahistine plus placebo, betahistine plus demethylbetahistine, betahistine plus diphenhydramine, betahistine plus α-methylhistamine, betahistine plus thioperamide, betahistine plus proxyfan, betahistine plus idazoxan, betahistine plus yohimbine, ciproxifan without betahistine) and underwent microsurgery as reported above. As soon as a clear picture could be taken, baseline measurements were recorded for 3 min. After the baseline measurements had been acquired, a 2-min infusion of the appropriate treatment was begun. Upon the beginning of the infusion, both cochlear blood flow and mean arterial pressure were recorded for 15 more minutes.

Statistical Analysis

Statistical analysis was carried out by Project R for Mac 3.0.0 GUI 1.60 Snow Leopard build (The R Foundation for Statistical Computing; http://www.r-project.org). Two-way analysis of variance (ANOVA) was used to detect significant differences; measurements of the treatment groups were compared with placebo at each given time point. In order to correct for multiple testing for different groups and time points, a Bonferroni t test was performed. A p value of $\alpha < 0.05$ was considered to be statistically significant.

Results

The Effect of Histaminergic H_1-Receptors on Cochlear Blood Flow and Normalized Cochlear Blood Flow

Infusion of betahistine together with demethylbetahistine, a histaminergic H_1-receptor agonist, caused a general drop in cochlear blood flow. From min 5 onwards, in which cochlear blood flow showed a brief increase, blood flow remained at a plateau around baseline level up to minute 11 (mean value for minutes 4–11 = 1.008 AU, standard deviation, SD = 0.116); from then on there was a strong tendency for blood flow to decrease. The average for the group receiving betahistine together with placebo in the same period of time was 1.180 AU (SD = 0.235). The lowest value was 0.766 AU at minute 18.

The group receiving betahistine together with the H_1-antagonist diphenhydramine showed no significant differences to the group that was treated with betahistine together with placebo.

None of the groups treated with betahistine plus diphenhydramine or betahistine showed a significant impact on normalized cochlear blood flow in comparison with the control group that was treated with betahistine together with a placebo (fig. 1, 2).

The Effect of Histaminergic H_3-Receptors on Cochlear Blood Flow and Normalized Cochlear Blood Flow

Infusion of the histamine H_3-receptor agonist α-methylhistamine showed significant differences in comparison with control from minutes 6 to 18. There was a general tendency of the cochlear blood flow to decrease under infusion of α-methylhistamine; the average value for minutes 6–18 was at 0.805 AU (SD = 0.225). The average for the placebo group in the same period of time was 1.219 AU (SD = 0.176).

The group receiving the histamine H_3-receptor antagonist thioperamide together with betahistine showed no major elevation from baseline; the changes in cochlear blood flow typical for betahistine were reversed. The mean value for minutes 4–18 was 0.994 AU (SD = 0.101). The values from minutes 7 to 18 are significantly different from the group receiving betahistine with placebo.

The same can be said about the group receiving the H_3-protean agonist proxyfan simultaneously with betahistine. Cochlear blood flow did not differ greatly from baseline throughout the entire observation. Minutes 8–18 differed significantly from the placebo group.

In the group that had received ciproxifan without betahistine, a H_3-selective inverse agonist/antagonist showed slightly increased cochlear blood flow. The average value for minutes 4–18 was 1.091 AU (SD = 0.063). Minutes 9–12 were significantly different from the betahistine group receiving solely betahistine.

No significant changes in normalized cochlear blood flow were observed in any group treated with betahistine together with histaminergic H_3-receptor agonists or antagonists in comparison with the control group (fig. 1, 2).

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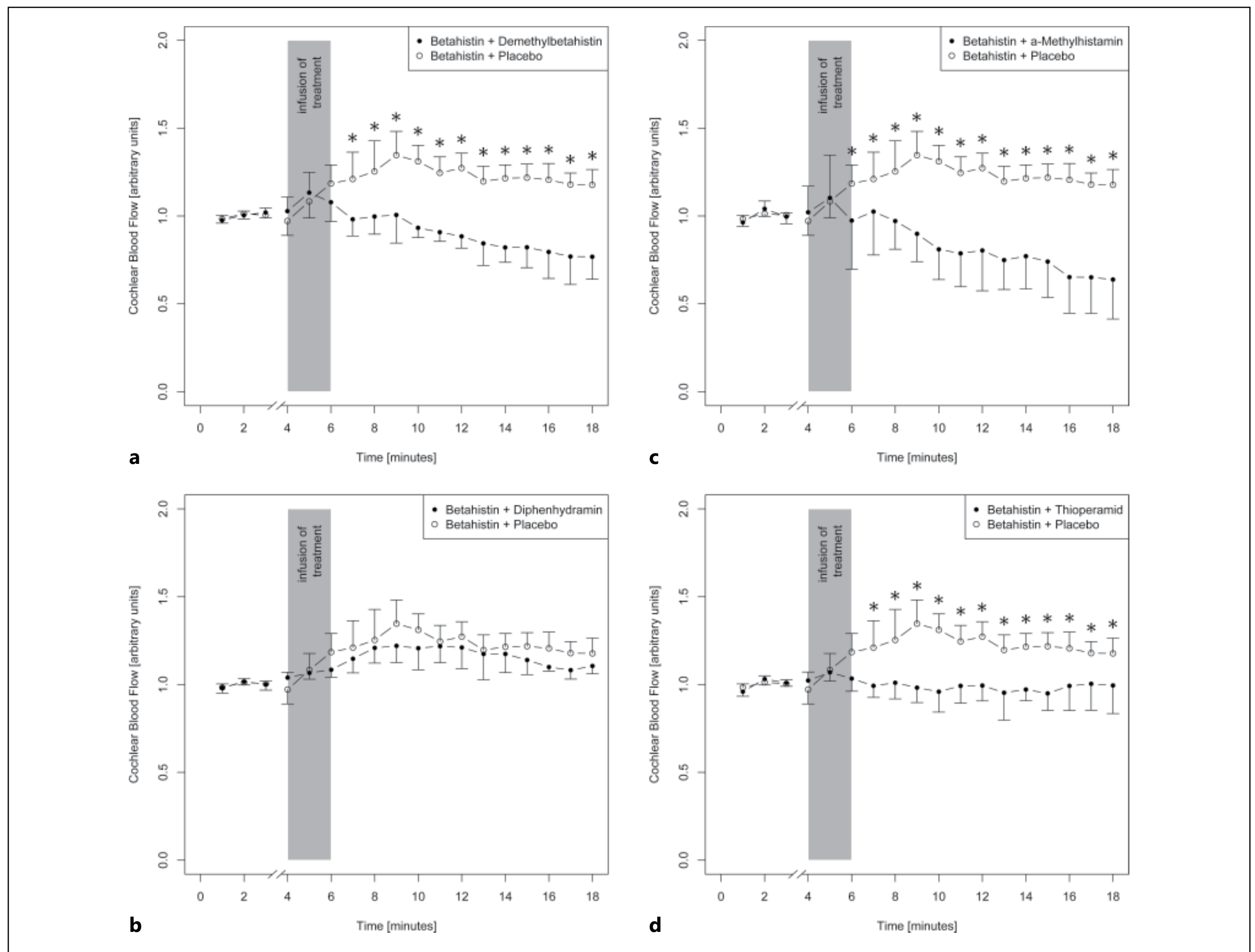

Fig. 1. Cochlear blood flow over time before and after infusion of betahistine together with treatment. **a** Betahistine plus demethylbetahistine. **b** Betahistine plus diphenhydramine. **c** Betahistine plus α-methylhistamine. **d** Betahistine plus thioperamide. **e** Betahistine plus proxyfan. **f** Ciproxifan. **g** Betahistine plus idazoxan. **h** Betahistine plus yohimbine. Data are presented as means ± SD. * $p < 0.05$. (*For figure 1e–h see next page.*)

The Effect of Adrenergic α_2-Receptors on Cochlear Blood Flow and Normalized Cochlear Blood Flow

The group receiving idazoxan showed a slight initial drop in cochlear blood flow. The lowest value at minute 4 was 0.889 AU (SD = 0.059). After a recovery up to minute 3, cochlear blood flow remained steady around baseline level. The average for minutes 7–18 was 1.011 AU (SD = 0.046). Minutes 5–17 were significantly different from the placebo group.

Infusion of betahistine together with yohimbine showed no change from basal values upon infusion or in the period thereafter. Cochlear blood flow in minutes 7–16 was significantly different from cochlear blood flow in the group receiving betahistine together with placebo.

None of the groups treated with betahistine and adrenergic α_2-receptor antagonists displayed significant changes in normalized cochlear blood flow in comparison with the group receiving betahistine with placebo (fig. 1, 2).

The Effect of H_1-Receptors on Mean Arterial Pressure

The group that was treated with demethylbetahistine showed an initial, yet steep, rise with a peak at minute 5 of 1.374 AU (SD = 0.496). From then on, blood pressure showed a general tendency to decrease. Significant differ-

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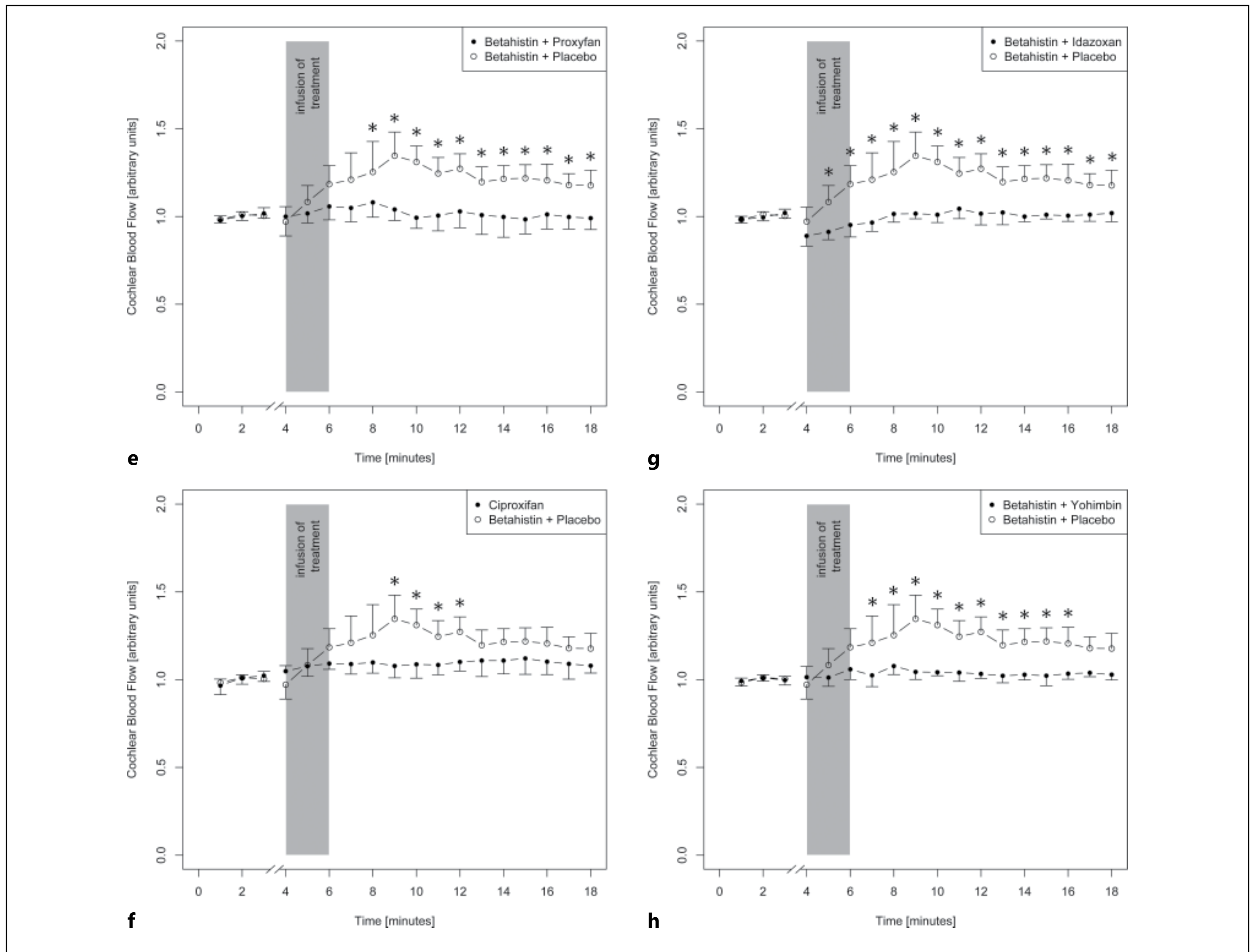

ences from placebo were detected at minutes 5 and 9–18. The group receiving diphenhydramine showed no significant differences from the control group (fig. 3).

The Effect of H_3-Receptors on Mean Arterial Pressure

Infusion of α-methylhistamine caused a steep increase for minutes 4–6. The peak was at minute 5 at 1.271 AU (SD = 0.296). From then on, blood pressure gradually declined to 0.556 AU (SD = 0.222). The arterial pressure was statistically different from the control group at minutes 5 and 8–18.

Treatment with betahistine in combination with thioperamide reversed the betahistine-typical changes and caused blood pressure to remain close to basal values. Significant differences from the control group were monitored at minutes 8–11.

The group receiving proxyfan together with betahistine showed similar effects to the aforementioned, meaning little deviation from baseline. Moreover, there was an overall tendency for blood pressure to decrease; in comparison with the control group, values at minutes 9 and 10 were significantly different.

Treatment with only ciproxifan led to no significant changes in blood pressure compared with the control group (fig. 3).

The Effect of Adrenergic α_2-Receptors on Arterial Blood Pressure

Infusion of betahistine in combination with idazoxan caused an initial, slight drop in blood pressure, while overall there was little change from basal values. In comparison with the group receiving betahistine with saline

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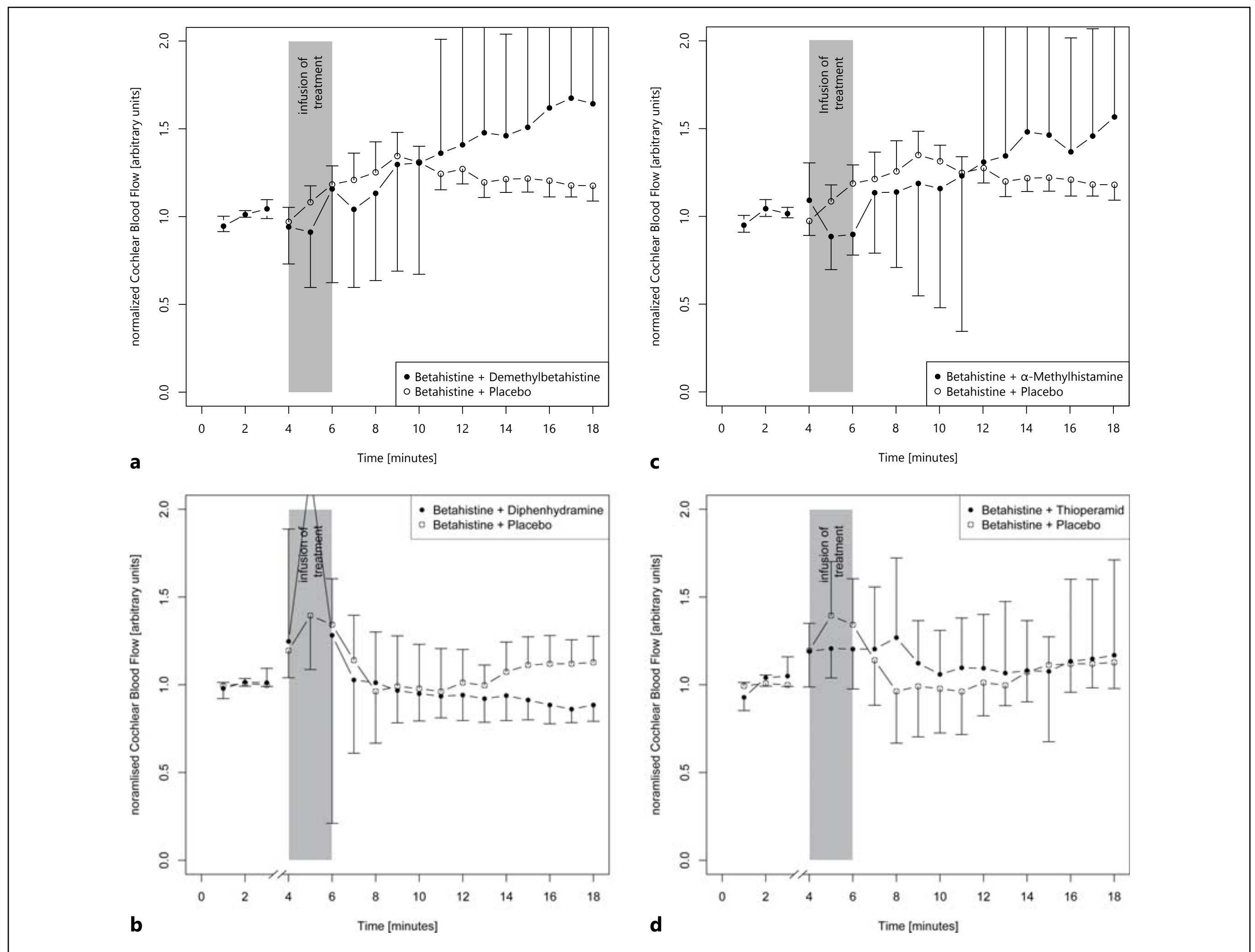

Fig. 2. Normalized cochlear blood flow over time before and after infusion of betahistine together with treatment. **a** Betahistine plus demethylbetahistine. **b** Betahistine plus diphenhydramine. **c** Betahistine plus α-methylhistamine. **d** Betahistine plus thioperamide. **e** Betahistine plus proxyfan. **f** Ciproxifan. **g** Betahistine plus idazoxan. **h** Betahistine plus yohimbine. Data are presented as means ± SD. * $p < 0.05$. (*For figure 2e–h see next page.*)

solution, values for minutes 8–11 were significantly different.

Treatment with yohimbine caused a similar effect, with an initial slight drop and the overall tendency for blood pressure to stay close to basal values. Comparison with the control group showed minutes 8, 9 and 10 to be significantly different (fig. 3). See supplementary table 1 for the effects of all histaminergic receptors. For an overview of the mechanism of action, structure, receptor affinities, and dosages of receptor agonists and antagonists used, see online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000368293).

Discussion

Betahistine is known to act as a weak agonist on the H_1-receptor [Gbahou et al., 2010]. Assuming that the increase in cochlear blood flow is mediated through the H_1-receptor, one would expect betahistine in combination with an H_1-selective agonist like demethylbetahistine [Arai and Chiba, 1999] to cause an increase in cochlear blood flow at least comparable in extent with that of betahistine alone. In turn, one would expect treatment with an H_1-receptor antagonist like diphenhydramine to reverse the increase in cochlear blood flow.

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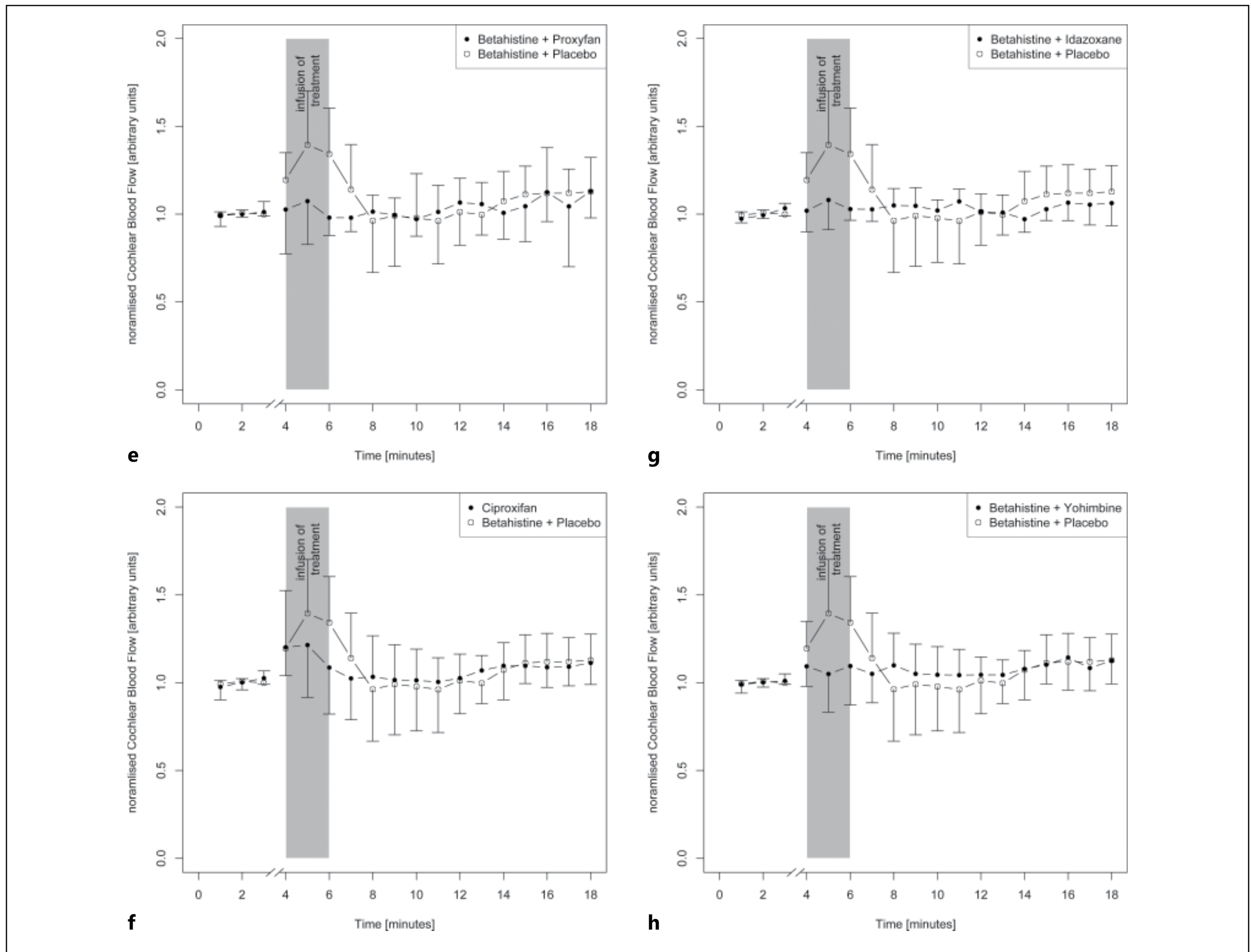

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However, infusion of betahistine and demethylbetahistine caused a drop in mean arterial pressure and cochlear blood flow. It has previously been described before both betahistine and demethylbetahistine are capable of reducing blood pressure considerably [Tobia et al., 1974]. Overall, the data presented here concerning demethylbetahistine could be a result of the progressive failure of cochlear blood flow autoregulation due to the continuously decreasing mean arterial pressure [Brown and Nuttall, 1994]. During minutes 4–11 cochlear blood flow is most likely to be in a steady state – maintained by autoregulation – whilst from minute 11 onwards, cochlear blood flow decreases owing to the failure of autoregulation due to the systemic decline of blood pressure. This view is further supported by the increasing values of normalized cochlear blood flow seen from minute 9 onwards. With these assumptions in mind, it seems improbable that the H_1-agonism of betahistine plays a major role in the mediation of betahistine effects. Fittingly, the group treated with the H_1-antagonist diphenhydramine yielded no significant differences from the control group in terms of cochlear blood flow or arterial pressure in the present study. These findings are in line with the literature that suggests that the H_1-receptor has no effect on betahistine-induced effects on cochlear blood flow [Laurikainen et al., 1993]. However, one more observation should be pointed out here: in previous experiments it has been shown that higher doses of betahistine show a significant yet short-lived drop in mean arterial pressure and cochlear blood flow at the beginning of betahistine infusion [Ihler et al., 2012a]. This initial and brief drop seems to be steeper the high-

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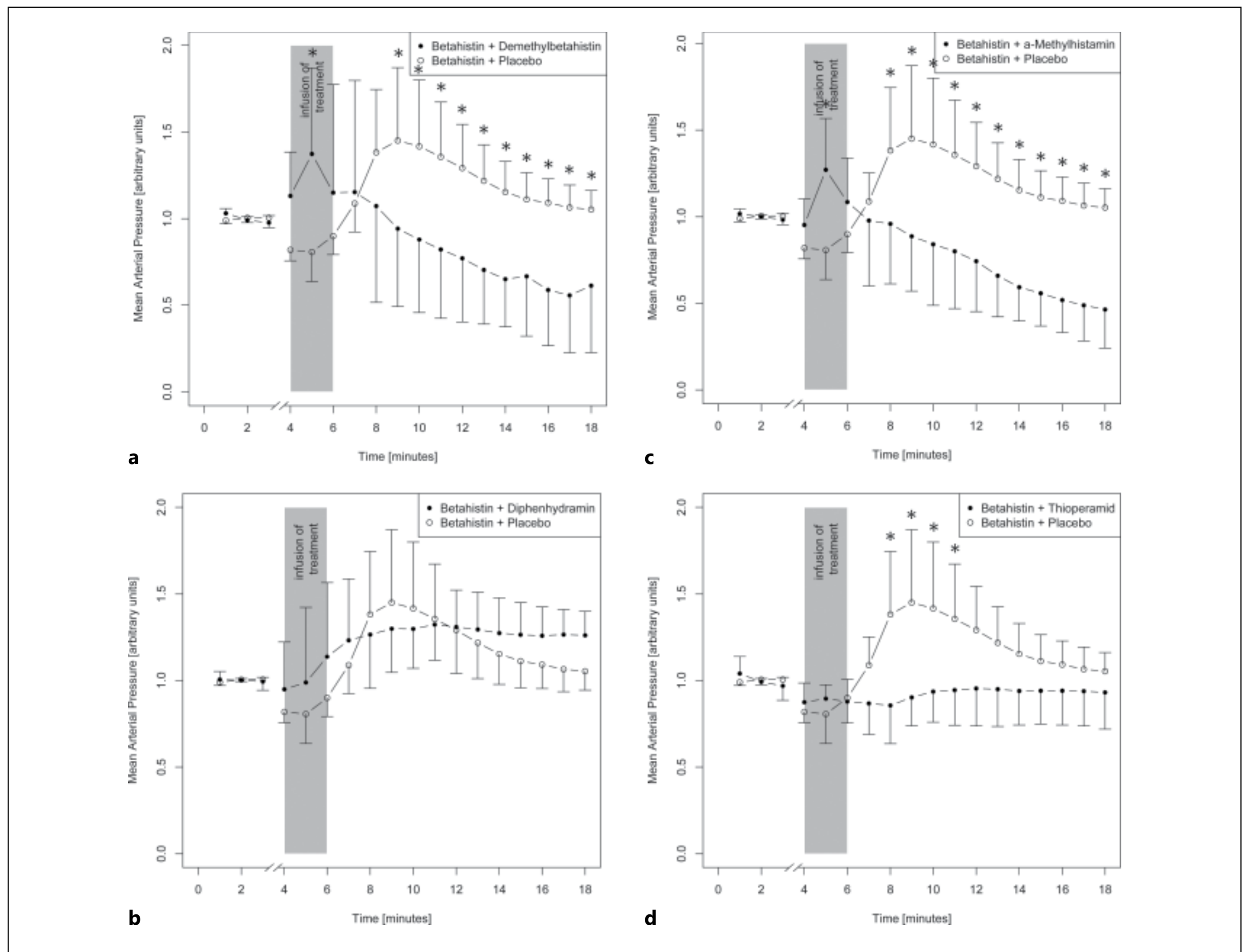

Fig. 3. Mean arterial pressure over time before and after infusion of betahistine together with treatment. **a** Betahistine plus demethylbetahistine. **b** Betahistine plus diphenhydramine. **c** Betahistine plus α-methylhistamine. **d** Betahistine plus thioperamide. **e** Betahistine plus proxyfan. **f** Ciproxifan. **g** Betahistine plus idazoxan. **h** Betahistine plus yohimbine. Data are presented as means ± SD. * $p < 0.05$. (*For figure 3e–h see next page.*)

er the concentration of betahistine [Dziadziola et al., 1999]. A similar although smaller drop (owing to our relatively low dosage of betahistine) was observed in the data presented here. The results of the diphenhydramine group suggest that this initial drop could potentially be reversed by the application of an H_1-antagonist such as diphenhydramine. Bearing in mind the previous assumption that the H_1-agonism of betahistine is most likely not involved in the increase of cochlear blood flow, it seems very possible that it is involved in the mediation of this initial drop in mean arterial pressure. These findings are in line with recent receptor affinity studies that pointed out that betahistine is very potent at the H_3-receptor and somewhat weaker at the H_1-receptor [Fossati et al., 2001; Gbahou et al., 2010], raising the idea that side effects of betahistine, like the aforementioned drop in mean arterial pressure and cochlear blood flow, could be H_1-mediated. This view is supported by the fact that typical betahistine side effects are also typically H_1-receptor-related reactions, including flushing, headaches, skin reactions, and low blood pressure [Parsons, 1991; Jeck-Thole and Wagner, 2006].

Betahistine acts as a potent inverse agonist at the H_3-receptor [Gbahou et al., 2010]. An inverse agonist is a li-

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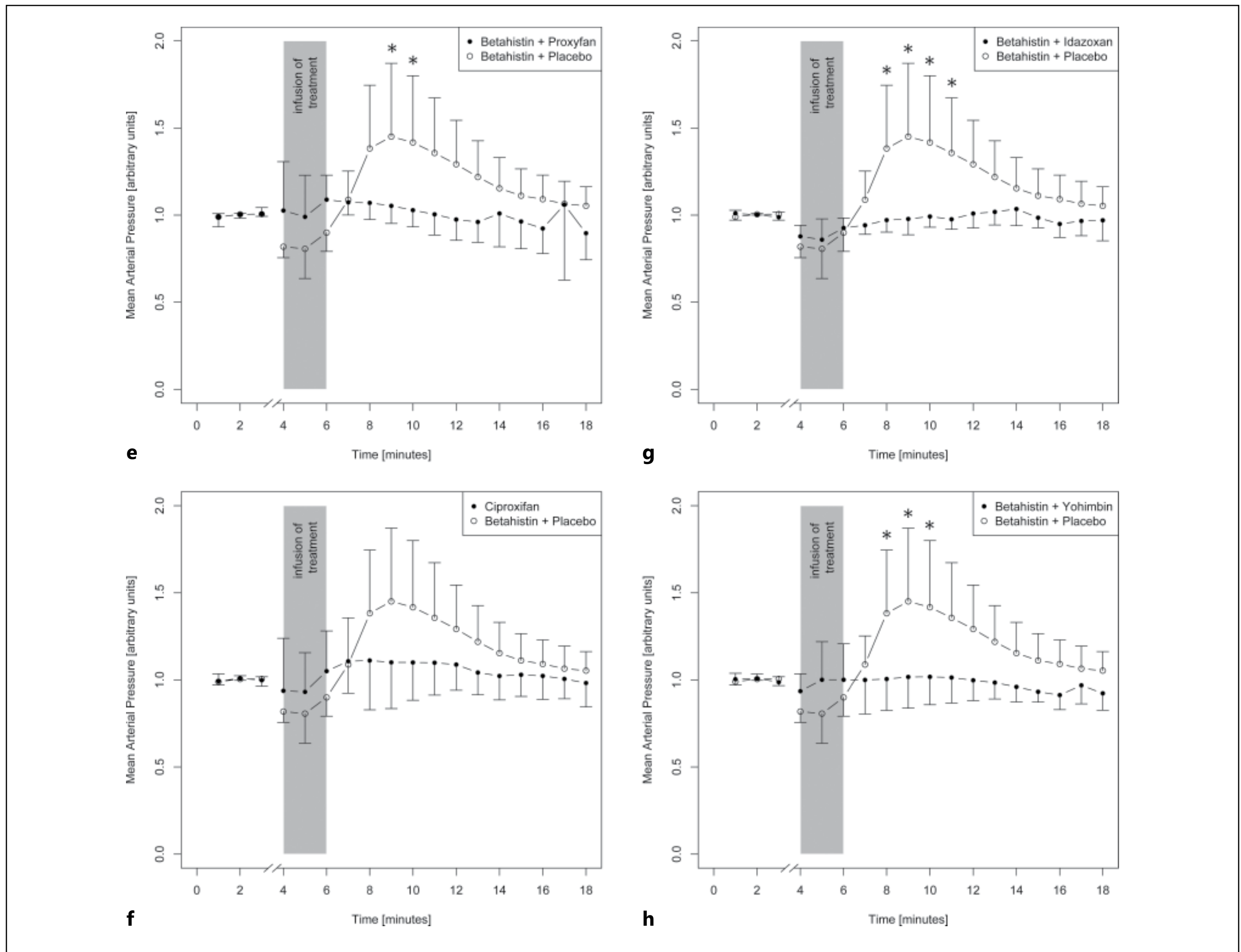

gand that binds to a receptor and decreases its constitutive activity [Kenakin and Williams, 2014]. Blocking of the H_3-receptor with proxyfan or thioperamide caused the suppression of changes in cochlear blood flow typically mediated by betahistine. The suppression of betahistine-induced changes in cochlear blood flow by the blockage of the H_3-receptor has previously been reported [Dziadziola et al., 1999] and was also observed in the present study. This indicates an involvement of the H_3-receptor in betahistine-induced changes in cochlear blood flow. The fact that infusion of betahistine together with the H_3-receptor agonist α-methylhistamine, which acts as an opponent on this receptor in comparison with betahistine, caused a significant and lasting drop in both cochlear blood flow and mean arterial pressure further supports this theory. The fact that α-methylhistamine in combination with betahistine decreases cochlear blood flow and arterial blood pressure has not been reported so far and contradicts a study that conducted a similar experiment [Laurikainen et al., 1998]. In this study it had been proposed that α-methylhistamine had no effect whatsoever on cochlear blood flow or blood pressure. However, in the aforementioned study, α-methylhistamine dosaging had been more than 10-fold lower, whilst betahistine concentrations were 15 times higher than in this setting, resulting in an agonist-to-betahistine ratio of over 150 times lower than in the experiments reported here. Hence, the overall results indicated a probable involvement of the histamine H_3-receptor in betahistine effects on cochlear blood flow. In order to elucidate this theory, one group was treated solely with ciproxifan, a competitive H_3-inverse agonist [Motawaj and Arrang, 2011]. Infusion of

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ciproxifan caused a moderate increase in both cochlear blood flow and mean arterial pressure – however, not to an extent comparable with that of betahistine. A possible reason for this finding could be a relatively low affinity to adrenergic α_2-receptors, which also seem to be involved in the mediation of betahistine-induced effects on cochlear blood flow and mean arterial pressure. Finally, even though ciproxifan has a lower K_i value than betahistine at the histaminergic H_3-receptor, and thus a greater affinity, this does not imply a stronger effect on the intracellular signaling cascades controlled by H_3-receptors.

Taking into account all of the above considerations, it seems likely that the histamine H_3-receptor plays a major role in the observed betahistine effects on cochlear blood flow.

It has been suggested several times that betahistine effects are not only mediated by histamine receptors, but that another class of receptors is involved as well. Candidates for this second receptor class have included acetylcholine [Laurikainen et al., 1993], imidazole [Laurikainen et al., 1998] and adrenergic [Laurikainen et al., 1998] receptors. It has been reported that pretreatment of animals with idazoxan, a potent adrenergic α_2-receptor antagonist, is capable of entirely reversing the betahistine-induced changes in cochlear blood flow [Laurikainen et al., 1998]. To the best of our knowledge, there have been no in vivo or in vitro investigations on the extent to which betahistine exerts an effect on α_2-receptors. In the presented data, betahistine effects were reversed by simultaneous infusion of both idazoxan, an α_2-/I_2-receptor antagonist, and yohimbine, an α_2-/5-HT_3-antagonist, together with betahistine. Overall, the fact that blockage of the α_2-receptor can also reverse betahistine changes similar to proxyfan and thioperamide suggests a noteworthy involvement of adrenergic α_2-receptors in betahistine effects too. This view is further supported by the fact that betahistine was originally discovered as a drug while searching for adrenergic properties of pyridylalkylamines [Hunt and Fosbinder, 1942].

The fact that both the α_2- and the H_3-receptor obviously play a major role in the mediation of betahistine effects raises a new question: do both receptors contribute directly to the increase in cochlear blood flow or could it be that they function as heteroreceptors that influence each other. Overall, the latter theory seems somewhat more likely, bearing in mind the fact that H_3-receptors are known to have a significant impact on systemic and local noradrenaline release [Malinowska et al., 1998; Mazenot et al., 1999]. Moreover, it has been shown that H_3-receptors are capable of interacting both with histaminergic and autonomic receptors in the periphery [Ishikawa and Sperelakis, 1987].

Taking this assumption even further, it could be postulated that the effects of betahistine at the cochlea are mere downstream effects caused by the increased blood pressure. Such a view could be supported by the fact that the cochlea lacks short-term autoregulation when systemic blood pressure increases [Vass et al., 1993], and that even successful betahistine therapy has failed to show a considerable impact on the endolymphatic hydrops on Ménière's patients [Gurkov et al., 2013]. Fittingly, none of the groups presented in this study happened to show a significant impact on normalized cochlear blood flow. However, it has also been shown that betahistine has a direct effect on vessels [Laurikainen et al., 1998; Santos-Silva et al., 2009]. In addition to that, a study conducted by this workgroup managed to show a significant increase of cochlear blood flow caused by the infusion of aminoethylpyridine, a product of betahistine metabolism [Bertlich et al., 2014]. At the same time, aminoethylpyridine had the tendency to lower mean arterial pressure, suggesting that betahistine effects are at least partially specific to the cochlear capillary network.

Conclusion

Betahistine effects seem to be mediated through histamine H_3-receptors. Furthermore, the data presented here indicate an involvement of the adrenergic α_2-receptors. The exact role of the adrenergic α_2-receptors could be explained with the heteroreceptor properties of the H_3-receptor.

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Disclosure Statement

The authors M. Bertlich, F. Ihler, S. Freytag, B.G. Weiss, and M. Canis declare that they have no conflicts of interest. Prof. M. Strupp declares to have received funds in return for consulting services to Abott, Pierre-Fabre and Biogen Idec as well as having received funds for the preparation of scientific training for Abbott, Biogen Idec, CSC, Henning Pharma, and GSK.

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Cochlear Pericytes Are Capable of Reversibly Decreasing Capillary Diameter In Vivo After Tumor Necrosis Factor Exposure

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Objective: The aim of this work was to evaluate the effect of tumor necrosis factor (TNF) and its neutralization with etanercept on the capability of cochlear pericytes to alter capillary diameter in the stria vascularis.
Methods: Twelve Dunkin–Hartley guinea pigs were randomly assigned to one of three groups. Each group was treated either with placebo and then placebo, TNF and then placebo, or TNF and then etanercept. Cochlear pericytes were visualized using diaminofluorescein-2-diacetate and intravasal blood flow by fluorescein-dextrane. Vessel diameter at sites of pericyte somas and downstream controls were quantified by specialized software. Values were obtained before treatment, after first treatment with tumor necrosis factor or placebo and after second treatment with etanercept or placebo.
Results: Overall, 199 pericytes in 12 animals were visualized. After initial treatment with TNF, a significant decrease in vessel diameter at sites of pericyte somas (3.6 ±4.3%, n = 141) compared with placebo and downstream controls was observed. After initial treatment with TNF, the application of etanercept caused a significant increase (3.3 ±5.5%, n = 59) in vessel diameter at the sites of pericyte somata compared with placebo and downstream controls.
Conclusion: We have been able to show that cochlear pericytes are capable of reducing capillary diameter after exposition to TNF. Moreover, the reduction in capillary diameter observed after the application of TNF is revertible after neutralization of tumor necrosis factor by the application of etanercept. It seems that contraction of cochlear pericytes contributes to the regulation of cochlear blood flow. **Key Words:** Capillary pericytes—Cochlear blood flow—Etanercept—Microcirculation—Pericytes—Tumor necrosis factor.

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Local blood flow is a critical parameter for cochlear function. Impairment of cochlear blood flow itself or its regulation has been associated with numerous pathologies of the inner ear, including, but not limited to, sudden sensorineural hearing loss (1–3), noise-induced hearing loss (4) or Ménière's disease (5–7).

To this point, it has commonly been assumed that capillary blood flow of most tissues is mainly regulated by the precapillary small arteries and arterioles (8), like the spiral modiolar artery in the cochlea (1). However, the role of capillary pericytes has recently been re-evaluated in numerous tissues. Pericytes are, generally speaking, cells that adhere to the outer walls of the capillaries (9) and fulfil a broad range of functions. They form physical barriers, like the blood–brain (10) or the blood–retina (11) barrier, play a role in tissue regeneration (12), and contribute to the stabilization of microvasculature (13,14). Moreover, pericytes are, at least partially, able to contract and thus decrease capillary diameter (9,14–17), eventually contributing to the short-term regulation of local blood flow (9,18–20).

Especially, the capillary pericytes of the brain have been subject to recent investigations: it has been found that neuronal capillary pericytes are not only among the first structures to actively increase local blood flow in times of increased oxygen demand by dilation (16), but also to be among the first cells to suffer from hypoxemia and thus contribute to a persistent decrease in local microcirculation in cerebral ischemia (17,19–21).

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It has been accepted that decreases in cochlear blood flow are the common final pathophysiological pathway of numerous inner ear diseases (1–3,22–24). Many of these pathologies seem to rely on the tumor necrosis factor (TNF) pathway to mediate their effects, including sudden sensorineural hearing loss (1,25) and noise-induced hearing loss (4,25). Moreover, there have been numerous reports that antagonization of tumor necrosis factor by the application of etanercept, a fusion protein that is used to competitively bind the tumor necrosis factor receptors (26,27), is considered to be beneficial in some of these pathologies (1,4,25,28).

Hence, we decided to investigate the effect of tumor necrosis factor on cochlear pericytes, their ability to affect capillary diameter and the potential of etanercept in revoking aforementioned effects.

METHODS

All of the experiments reported were approved according to local regulations by the responsible authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit LAVES, Oldenburg, Federal Republic of Germany) under the license no. 33.9-42502-04-14/1427.

Animals

Animals used were albino Dunkin–Hartley guinea pigs specifically bred for experimental use and were purchased from authorized retailers (Harlan Laboratories, Ober-Ramstadt, Hesse, Germany and Charles River Laboratories, Sulzfeld, Germany). The body weight ranged from 200 to 450 g. Anesthesia was induced by an intraperitoneal injection of 50 mg/kg bodyweight (b.w.) ketamine and 5 mg/kg b.w. xylazine and sustained by repeated intramuscular injections of 25 mg/kg b.w. ketamine and 2.5 mg/kg b.w. xylazine every 30 minutes.

Surgical preparation lasted approximately 90 to 120 minutes. After the experiments were conducted, animals were euthanized by an overdose of anesthesia and subsequent cervical dislocation.

Surgical Approach

Intravital microscopy using fluorescein-labeled dextrane for the investigation of cochlear microcirculation was initially described in 1987 by Nuttal (29). He was also among the first to describe diaminofluorescein-2-diacetate as a selective marker for cochlear pericytes (15).

After the induction of anesthesia, a cervical venous catheter was surgically implemented. The external ear and bone covering the auditory bulla were carefully removed. By doing this, a free view on the lateral cochlear wall was achieved. After this, small periosteal vessels were removed using a microsponge. After the removal of the vessels, a small rectangular window of approximately 500×500 μm was carved into the cochlea at the second turn, exposing the stria vascularis. Afterward, fluorescein-labeled dextrane (molecular weight 500,000; 0.05–0.1 ml of a 5% solution in 0.9% NaCl; Sigma-Aldrich, Deisenhofen, Germany) was applied intravenously, allowing direct visualization of intravascular blood flow in the stria vascularis under illumination with a Leica EL6000 light source (Leica Microsystems, Wetzlar, Germany). After a clear view of the vessel window had been obtained, the bulla was filled with a solution of 5 mM 4,5-diaminofluorescein diacetate in dimethyl sulfoxide diluted 1:10 with sterile saline solution. After 20 minutes, the bulla was repeatedly washed with sterile saline, and images were obtained. If at least six pericytes were clearly identified, treatment continued; otherwise, the experiment was aborted. Images were obtained with a Leica M205 FA stereomicroscope (Leica Microsystems, Wetzlar, Germany). The proprietary Leica Application Suite software was used to record and save images for later off-line analysis. Quantification of blood velocity and vessel diameter was done by Cap-Image (Dr. Zeintl Biomedical Engineering, Heidelberg, Germany), a software specifically designed for this purpose (30). A schematic of where capillary diameter was measured can be observed in Fig1C and A sample of the videos recorded is available with the online supplemental material (http://links.lww.com/MAO/A545).

Treatment Protocol

Twelve animals were randomly assigned to one of three groups. A schematic of the general course of the experiment as well as the treatment of the individual groups can be observed in Figure 1A and B. After a clear image of the stria vascularis was obtained and a minimum of six pericytes were visible, basal values were recorded. After that, either tumor necrosis factor or a placebo were applied topically for 20 minutes. After the application, the bulla and stria vascularis were rinsed for approximately 10 minutes and images were obtained again.

Finally, either etanercept or a placebo were topically applied for 20 minutes again, the bulla was then rinsed with sterile saline for 10 minutes and afterward, final images were obtained. After the acquisition of the final images, the animals were euthanized.

Statistics

At three timepoints (basal values, after first treatment, after second treatment), which were 30 minutes apart from each other, images of the stria vascularis with visualized pericytes were obtained. During off-line analysis, two parameters were quantified: the capillary diameter at each site of a pericyte soma (μm) and capillary diameter downstream of these pericyte somas (μm) as a control (see also Fig. 1C). The values reported are relative change in capillary diameter compared with baseline $\pm$ standard deviation. The absolute values for the capillary diameters can be found in the online supplemental material (http://links.lww.com/MAO/A547).

To detect significant differences, we fitted linear mixed models that included a random effect for the animal and were estimated using a restricted maximum likelihood approach. A p value < 0.05 was considered to be significant. The software used for this was Project R (Build 3.2.5 for Windows, The R Project for Statistical Computing, http://www.r-project.org/).

RESULTS

Capillary Diameter at Sites of Somas of Pericytes and Downstream Controls After Initial Application of Tumor Necrosis Factor or Placebo

Overall, $n = 199$ pericytes measured in 12 animals were considered. Of these, 141 pericytes were treated with tumor necrosis factor and 58 were treated with placebo; this disparity is owed to the fact that eight animals in two groups were initially treated with TNF. Since the treatment was biologically the same, the group were pooled.

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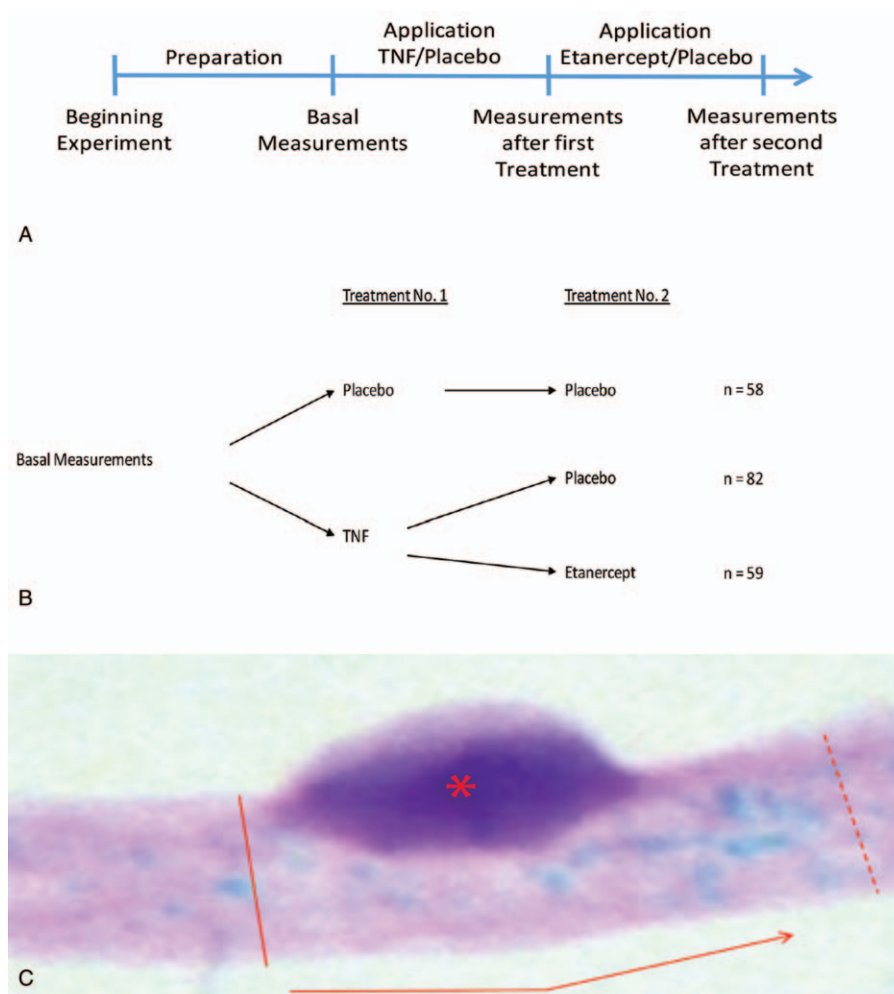

FIG. 1. *A,* Schematic of the timeline of each individual experiment. *B,* Distribution of the guinea pigs into individual groups. Numbers on the right indicate the number of pericytes measured in each group. *C,* Schematic of how diameters were measured at pericyte sites (continuous line) and downstream controls (dotted line). * indicates pericyte soma, the *arrow* indicated the direction of blood flow.

After the application of placebo, the vessel diameter at pericyte sites remained the same, only marginally increasing by $0.3 \pm 2.0\%$ while treatment with tumor necrosis factor resulted in a decrease in diameter at sites of pericytes of $3.6 \pm 4.3\%$. At downstream control sites, application of placebo lead to a marginal increase of diameter of $0.4 \pm 2.5\%$ while application of tumor necrosis factor caused a diameter decrease of $2.3 \pm 2.9\%$. The fitted linear models showed a significant difference between treatment (TNF) and placebo ($p < 0.001$). There was also a significant ($p = 0.002$) difference between sites of pericyte somas and downstream controls (Fig. 2).

Fraction of Contractile Pericytes After Initial Application of Tumor Necrosis Factor

Of the 141 pericytes that were initially treated with tumor necrosis factor, 39 (27.7%) showed a decrease in diameter that was >8.0% from the initially recorded basal value.

Capillary Diameter at Sites of Somas of Pericytes and Downstream Controls After Application of Etanercept or Placebo

Overall, 199 pericytes were considered. Of these 199, 58 pericytes in four animals had been treated with placebo twice, and 141 pericytes in 8 animals had initially been treated with TNF. Of these 141 pericytes, 82 were treated with placebo after the application of TNF, while 59 were treated with etanercept after initial TNF treatment.

Twofold application of placebo caused a negligible increase of diameters of $0.0 \pm 2.7\%$ at pericyte soma sites as well as a marginal increase of $0.2 \pm 2.7\%$ at respective downstream control sites.

Application of placebo after the application of tumor necrosis factor caused a minimal increase of diameters of $0.4 \pm 2.4\%$ at pericyte soma sites and an insignificant decrease of $0.4 \pm 2.5\%$ at respective downstream control sites.

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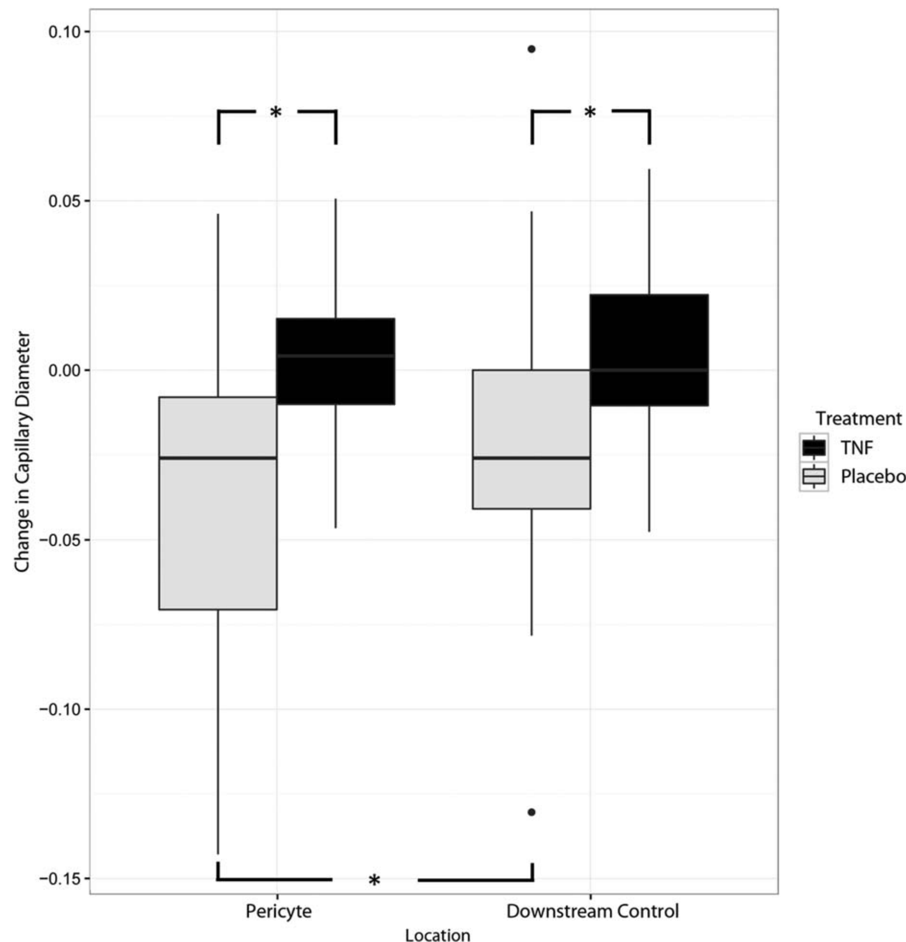

FIG. 2. Relative diameter change compared with baseline at sites of pericyte somas and downstream controls after initial application of tumor necrosis factor or placebo. • = outliers * = $p < 0.05$.

Finally, after the application of etanercept following the application of TNF, an increase in diameter of $3.3 \pm 5.5\%$ was observed at sites of pericyte somas. Downstream controls showed an increase of $1.8 \pm 5.5\%$.

The fitted linear mixed models showed a significant difference between treatment (Etanercept) and placebo ($p = 0.021$). We also demonstrated a statistically significant difference effect between treatment with pericytes and downstream controls ($p = 0.029$, Fig. 3).

DISCUSSION

First, we have been able to show that cochlear pericytes are capable of decreasing capillary diameter at sites of pericyte somas upon a physiological stimulus. Mere contractions of cochlear pericytes have already been shown by Dai et al. in 2009 (15). In this work, the proportion of pericytes that were able to show an active reduction of capillary diameter ranged from approximately 20 to 40%. Fittingly, our results are in line with the results reported.

However, in the aforementioned work, contractions were only observed under nonphysiological conditions such as very high extracellular concentrations of electrolytes like potassium or calcium. In contrast, this is the first time that a significant reduction of capillary diameter by cochlear pericytes has been shown after a physiological stimulus. The fact that tumor necrosis factor seems to play a major role in many inner pathologies, including acoustic (4) or physical (28) trauma as well as sudden sensorineural hearing loss (1) and that most of these pathologies coincide with impairment of cochlear blood flow (1,4) suggests that impairment of cochlear blood flow is at least partially mediated by active contractions of cochlear pericytes.

Since it has been shown that neutralization of tumor necrosis factor by etanercept (1,4,25) and blocking of sphingosine-1-phosphate (S1P) signaling (1,31) are effective in preventing tumor necrosis factor-related decreases in cochlear blood flow, it seems likely that the vascular effects of tumor necrosis factor on pericytes are at least partially mediated by S1P signaling. Fittingly, TNF-S1P-signaling has already been described to cause vascular contractions (32). In addition to this, defects in the sphingosine-1-phosphate receptor 2 are associated with a rapid degeneration of the stria vascularis as well as severe hearing loss, suggesting an integral role of sphingose-1-phosphate-signaling for stria vascularis preservation (33,34).

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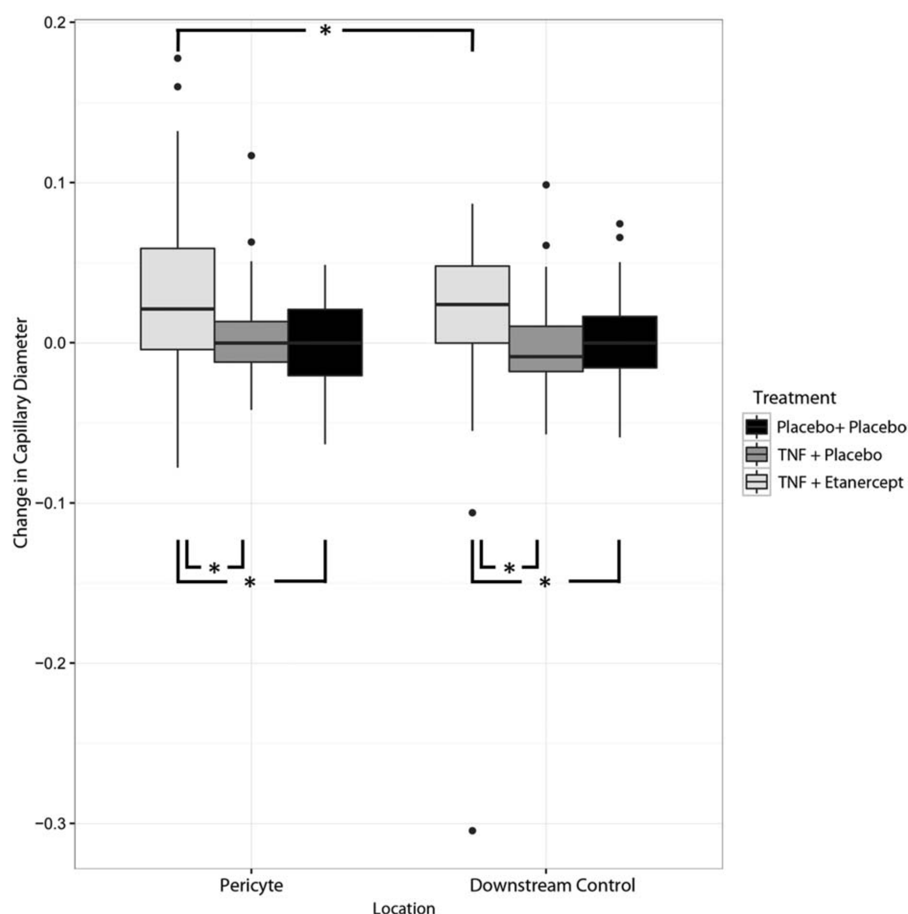

FIG. 3. Relative diameter change compared with baselines at sites of pericyte somas and downstream controls after the application of etanercept or placebo after the initial application of tumor necrosis factor. ● = outliers * = $p < 0.05$.

Moreover, we have been able to show that cochlear pericytes are not only able to actively reduce capillary diameter, but to dilate again if the stimulus for contraction (tumor necrosis factor) is neutralized by topical application of etanercept. The fact that cochlear pericytes are not only able to contract, but to relax again, is new and—to the best of our knowledge—has not been shown in any scientific publication so far. This observation is in line with previous studies that have found tumor necrosis factor-induced decreases in cochlear microcirculation can be revoked upon neutralization of tumor necrosis factor by application of etanercept (31).

In addition, clinical effects of successful therapy with etanercept are often associated with improved microcirculation (35–37).

Overall, the facts presented in our work strongly suggest that cochlear pericytes play an active role in regulating local blood flow, much alike the pericytes in other tissues.

Taking this assumption even further, one might postulate that cochlear pericytes have a very similar function to cerebral pericytes as early mediators of cochlear blood flow. Not only do cochlear pericytes show what seems to be a bidirectional control of capillary diameter at sites of pericyte somata like the cerebral pericytes do (16). Cochlear pericytes could also play a key role in one of the most common inner ear pathologies, sudden sensorineural hearing loss (SSNHL): it has often been postulated that SSNHL is of vascular origin, since it is like the retinal vein thrombosis, a pathology of clearly vascular origin, usually one sided and has similar risk factors (38). Increased fibrinogen levels have been known to reduce cochlear microcirculation and increase hearing threshold levels like it is observed in SSNHL (2). Decreased cochlear blood flow is also known to coincide with decreased pO_2 levels in the cochlea (3), suggesting a similar ischemic damage dealt to the cochlea and its microvasculature to that observed in the ischemic brain (17).

This view is further supported by the fact that we have shown etanercept to be capable of revoking previously induced contraction in cochlear pericytes. Since etanercept has also been suggested to be effective in the therapy of SSNHL (1), there is strong evidence that pericytes play a major role in SSNHL, possibly contributing to the continuous decrease in local microcirculation in a vicious cycle of ischemia and hypoemia.

In conclusion, we have been able to show that cochlear pericytes are capable of contraction, which has been reported previously. However, we have been able to

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show that this contraction takes places after the exposition to a physiological stimulus and that this contraction is clearly reversible. These new findings make it very likely that cochlear pericytes contribute to the regulation of cochlear blood flow, much alike the pericytes of the central nervous system, where they play an integral role in health and disease.

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4

Bertlich M., Ihler F., Freytag S., Weiss B., Jakob M., Strupp M., Canis M., *Fingolimod (FTY-720) is capable of reversing tumor necrosis factor induced decreases in cochlear blood flow*, Otol Neurotol, 2017 Sep;38(8):1213-1216

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Fingolimod (FTY-720) is Capable of Reversing Tumor Necrosis Factor Induced Decreases in Cochlear Blood Flow

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Hypothesis: The potential of Fingolimod (FTY-720), a sphingosine-1-phosphate analogue, to revoke the changes in cochlear blood flow induced by tumor necrosis factor (TNF) was investigated.
Background: Impairment of cochlear blood flow has often been considered as the common final pathway of various inner ear pathologies. TNF, an ubiquitous cytokine, plays a major role in these pathologies, reducing cochlear blood flow via sphingosine-1-phosphate-signaling.
Methods: Fifteen Dunkin-Hartley guinea pigs were randomly assigned to one of three groups (placebo/placebo, TNF/placebo, TNF/FTY-720). Cochlear microcirculation was quantified over 60 minutes by in vivo fluorescence microscopy before and after topical application of placebo or TNF (5 ng/ml) and after subsequent application of placebo or FTY-720 (200 μg/ml).
Results: Treatment with TNF led to a significant decrease of cochlear blood flow.
Following this, application of placebo caused no significant changes while application of FTY-720 caused a significant rise in cochlear blood flow.
Conclusions: FTY-720 is capable of reversing changes in cochlear blood flow induced by application of TNF. This makes FTY-720 a valid candidate for potential treatment of numerous inner ear pathologies. **Key Words:** Cochlear blood flow—Fingolimod—Microcirculation—Tumor necrosis factor.
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Local microcirculation is a critical parameter for cochlear function. Impairment of microcirculation results in decreases in partial oxygen pressure (1) and subsequent loss of function, including significant decreases in endocochlear potential and increases in hearing thresholds (2–4). Impairment and regulation of cochlear microcirculation also plays a role in the pathophysiology and treatment of other pathologies such as Menière's disease (5) and inflammatory inner ear pathologies (3,6).

Tumor necrosis factor (TNF) is an ubiquitous cytokine that is involved in various types of disease, including, but not limited to malignancies (7), autoimmune diseases (8), and depression (9). It has also been established that TNF is capable of impairing local circulation in various tissues, including the cochlea (6,10). Moreover, several studies have been able to demonstrate that TNF is involved in a number of inner ear pathologies, such as sudden sensorineural hearing loss (6), acoustic (3), and physical trauma (11).

Research has shown that the effects of TNF are mediated by several pathways (12): after binding to its specific receptor, TNF amplifies intracellular signaling pathways that lead to induction of cell death, including NFκB- and JNK-signaling as well as caspases. TNF also activates sphingosine-1-phosphate signaling (6). Overall, the short-term effects of TNF related pathologies are mediated by sphingosine-1-phosphate signaling (6) while the long-term effects are instead mediated by intracellular signaling leading to cell death, including NFκB- and JNK-signaling (13).

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All substances that have been used to reverse short-term effects of TNF on cochlear microcirculation have either not been specific to sphingosine-1-phosphate signaling (14) or have been experimental and have not been approved for human use (6). However, since 2010, Fingolimod (FTY-720), a sphingosine-1-phosphate analogue that is sold under the name Fingolimod has been approved for clinical use in human patients suffering from multiple sclerosis (15,16). Upon binding the sphingosine-1-phosphate receptor, it is actively transported into the expressing cell and thus cannot be activated anymore (17,18). Hence, we examined the potential of FTY-720 to reverse changes in cochlear microcirculation induced by topical application of TNF.

MATERIALS AND METHODS

Ethics Statement

All experiments were performed according to local state regulations and approved by the responsible authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit LAVES) under the animal license no: 33.9–42502–04–14/1427.

Animals

Animals were Dunkin-Hartley guinea pigs bred for experimental use and purchased from Harlan Laboratories (Ober-Ramstadt, Hesse, Germany), weighing 200 to 450 g. Anesthesia was induced by an intraperitoneal injection of 50 mg/kg bodyweight (b.w.) ketamine and 5 mg/kg b.w. xylazine and sustained by repeated intramuscular injections of 25 mg/kg b.w. ketamine and 2.5 mg/kg b.w. xylazine every 30 minutes.

Surgical preparation lasted approximately 60 to 90 minutes. After the experiments were conducted, animals were euthanized by an overdose of anesthesia and subsequent cervical dislocation.

Surgical Approach

The technique applied in this experiment has been previously described (19–21). After induction of anesthesia, a cervical venous catheter was surgically implemented. Subsequently, the external ear and the temporal bone covering the bulla were removed, thus exposing the cochlea. Overlying periosteal vessels were carefully removed using a microsponge. As soon as all the vessels were removed, a rectangular window was carved into the cochlea above the second turn, exposing the stria vascularis. Finally, fluorescein-labeled dextrane (molecular weight 500,000; 0.05–0.1 ml of a 5% solution in 0.9% NaCl; Sigma–Aldrich, Deisenhofen, Germany) was applied intravenously, allowing direct visualization of intravascular blood flow in the stria vascularis, with a contrast between erythrocytes and plasma. Following illumination with a Leica EL6000 light source (Leica Microsystems, Wetzlar, Germany), images were obtained with a Leica M205 FA stereomicroscope. The proprietary Leica Application Suite software was then used to process and save the generated data for later off-line analysis. An example of the video material acquired is available with the supplementary digital content (http://links.lww.com/MAO/A538).

Velocity (micrometers per second) and diameter (micrometers) of stria vascularis vessels were quantified using Cap-Image (Dr. Zeintl Biomedical Engineering, Heidelberg, Germany), a software specifically designed for this purpose (22). An exact description with sample pictures of the digital quantification of intravascular blood velocity is also available with the supplementary digital content (http://links.lww.com/MAO/A539).

For each vessel the intravascular blood flow was calculated with a formula proposed by Wayland: $q = (v/1.6) \times (d/2)^2 \times \pi$ (23). To correct for interindividual differences as well as differences between individual vessels, units are reported as arbitrary units (AU), representing change from the initially acquired basal values.

Treatment Protocol

Five animals were randomly assigned to one of three groups (placebo/placebo, TNF/placebo, or TNF/FTY-720). After the surgical preparation and before treatment had begun, basal images were acquired for later analysis. Following this, placebo or TNF was applied topically for 20 minutes. Afterwards, the bulla was rinsed with 0.9% saline solution for 10 minutes and the microcirculation was quantified again. Then, placebo or FTY-720 was applied for 20 minutes and the bulla was washed again before final images were acquired. Similar protocols have been used in previous experiments addressing this topic (10,14). After the protocol had been finished, the animals were euthanized.

For placebo, a sterile saline solution was chosen. The concentration of TNF was 5.0 ng/ml in sterile saline solution as reported in previous experiments (10,14). For the FTY-720 solution, 1 mg of FTY-720 was dissolved in 5 ml of dimethyl sulfoxide, resulting in a concentration of 200 μg/ml. This was then diluted 1:10 in sterile saline solution, resulting in a final concentration of 20 μg/ml FTY-720 which was then applied.

Statistics

Statistical analysis was carried out using Project R (Build 3.3.2 for Windows, The R Project for Statistical Computing, http://www.r-project.org/). To detect differences between the groups as well as between the timepoints within the groups, we used a Wilcoxon test. This test allows paired and unpaired two-sample nonparametric comparisons. We used a Bonferroni correction to adjust for multiple testing, thus a p value of $\alpha < 0.005$ was considered to be statistically significant.

RESULTS

Effect of Placebo + Placebo on Cochlear Blood Flow

The initial topical application of placebo leads to no significant changes in cochlear blood flow, which remained steady at 1.01 ± 0.06 AU (Fig. 1, left column). Subsequent application of placebo leads to a drop in cochlear blood flow to 0.92 ± 0.09 AU. There were no significant differences within the group (Table 1). The values acquired after the first treatment with placebo were significantly different from those values obtained after treatment with TNF (Table 2).

Effect of TNF + Placebo on Cochlear Blood Flow

Initial application of TNF led to a drop in cochlear blood flow to $.81 \pm .07$ AU (Fig. 1, middle column). This was significantly different from the basal values as well as compared with placebo (Tables 1 and 2). Subsequent application of placebo induced no change in cochlear blood flow, which remained steady at 0.81 ± 0.10 AU.

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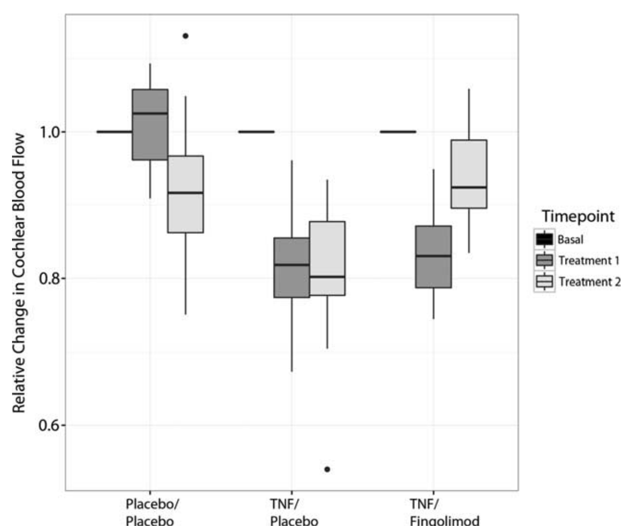

FIG. 1. Relative changes in cochlear blood flow over time, before initial treatment, in between treatments and after final treatment, reported in arbitrary units (AU).

This value was significantly different from the initial acquired basal value as well as the final values measured after application of TNF and subsequently FTY-720, but not significantly different from the values obtained within the same group after initial TNF application (Tables 1 and 2).

Effect of TNF + FTY-720 on Cochlear Blood Flow

Again, initial application of TNF caused a significant drop in cochlear blood flow to 0.83 ± 0.06 AU (Fig. 1, right column; Tables 1 and 2). Subsequent application of FTY-720 caused an increase in cochlear blood flow to 0.94 ± 0.07 AU. This increase was significantly different from the previously obtained values within this group (Table 1) as well as significantly different from the endpoint of the group that was treated with TNF and subsequently placebo, but not different from the values that were obtained in the group after the twofold application of placebo (Table 2).

DISCUSSION

In this study we confirmed that TNF decreases cochlear blood flow after topical application. This finding is in line with previous reports (6). Moreover, it has been reported that the effect of TNF is dose-dependent (14). It was previously also demonstrated that the effects of TNF on cochlear blood flow could be reversed by blocking the TNF receptor with etanercept (6,14). Etanercept has, so far, been shown to have beneficial effects in various cochlear pathologies that coincide with impairments of cochlear blood flow, including sudden sensorineural hearing loss (6) or acoustic trauma (3).

We demonstrated that topical application of FTY-720 is able to reverse the effects of TNF on cochlear blood flow: application of FTY-720 caused a significant increase in cochlear blood flow. The fact that the cochlear blood flow had also decreased somewhat in the group that had received a placebo twice could be explained by increased clotting in the capillaries caused by the surgical manipulation, a phenomenon that has previously been reported in this animal model (20). Even though this could be a potential confounder in the animal model used in this study, this also raises the threshold levels to obtain significant results, assuming that FTY-720 would increase cochlear blood flow compared with previous values.

The effect of FTY-720 is most likely mediated by its interaction with the sphingosine-1-phosphate-receptor. It has been known to bind the receptor, causing it to be transported into the inside of the cell, thus preventing it from being activated (17). This viewpoint is supported by the fact that blocking of the sphingosine-1-phosphate receptor has been able to revoke typical effects of TNF on microvasculature (6,10,24,25). Additionally, another potential mode of action has to be taken into account: FTY-720 has also been known to interact with the cannabinoid CB1-receptor; more specifically as a competitive CB-1-antagonist (26). It has been reported that antagonism at the CB-1-receptor is capable of preventing decreases in microcirculation caused by endotoxemia in other tissues, specifically the small intestine (27). This argument is further supported by the fact that endotoxemia is thought to cause several complications involving the TNF pathway and that blockage of the TNF-receptor using etanercept significantly reduces the immediate clinical effects of endotoxemia (28,29).

The presented results show that FTY-720 is capable of reversing the effects of TNF on cochlear blood flow. Assuming that alterations in cochlear blood flow cause a

TABLE 1. *p Values for the comparisons of time points in between the groups. Values marked bold were considered significant*

	Basal Versus Treatment 1	Basal Versus Treatment 2	Treatment 1 Versus Treatment 2
Placebo/placebo	$p = 0.561$	$p = 0.010$	$p = 0.005$
TNF/placebo	$p < \mathbf{0.001}$	$p < \mathbf{0.001}$	$p = 1.000$
TNF/FTY-720	$p < \mathbf{0.001}$	$p = 0.013$	$p < \mathbf{0.001}$

FTY-720 indicates Fingolimod; TNF, tumor necrosis factor.

TABLE 2. *p Values for the comparions of groups within time points. Values marked bold were considered significant*

	Treatment 1	Treatment 2
Placebo/placebo versus TNF/placebo		$p = 0.005$
TNF/placebo versus TNF/FTY-720	$p < \mathbf{0.001}$[a]	$p < \mathbf{0.001}$
Placebo/placebo versus TNF/FTY-720		$p = 0.539$

[a]The animals of the TNF/placebo and the TNF/FTY-720 group at Treatment 1 were pooled and compared against the animals that had received placebo, since the treatment both groups had received was biologically the same.

FTY-720 indicates Fingolimod; TNF, tumor necrosis factor.

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drop in partial oxygen pressure (1) and thus eventually ischemia and subsequent apoptosis of inner hair cells, FTY-720 might be a promising agent fit for clinical testing in various inner ear pathologies, including sudden sensorineural hearing loss (4,6), noise trauma (3), as well as physical trauma to the cochlea (11). As an additional benefit in comparison to other drugs that have been suggested as treatments for these pathologies, such as AM-111 (30) or JTE-013 (6), FTY-720 has already been approved for clinical use in relapsing remitting multiple sclerosis and, thus, has a well known safety profile. Moreover, its route of administration (oral intake, once daily) is significantly less complicated than the route of administration of etanercept, which has also been suggested as a potential treatment for these pathologies (3,6,14) and which has to be applied by daily subcutaneous injections.

Common side-effects of FTY-720 include leukopenia with the potential risk of opportunistic infections, bradycardia, elevation of liver enzymes, arterial hypertension, and macular edema (31). Since the need of an observational period of 6 hours after the first intake and the need of regular follow up examinations including frequent control of liver enzymes and lymphocytes during therapy with FTY-720, the intratympanic installation and subsequent crossing into the cochlea might be considered.

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5

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Role of capillary pericytes and precapillary arterioles in the vascular mechanism of betahistine in a guinea pig inner ear model

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A B S T R A C T

Aims: Betahistine is a histamine analogue that is used for the treatment of Menière's disease. Animal studies showed that it increases local blood flow in the stria vascularis. In terms of its mode of action, recent studies have prompted discussion of whether betahistine actively affects cochlear microcirculation by dilations of pericytes or of precapillary arterioles or by mere downstream effects. Hence, we investigated the effects of betahistine on cochlear capillary pericytes and precapillary arterioles.
Main methods: The stria vascularis was visualized in 12 guinea pigs by in vivo fluorescence microscopy. In these, 152 pericytes were stained and local diameter at sites of pericyte somas and downstream controls as well as intravascular blood flow were measured before and after betahistine application. Moreover, in two guinea pigs the precapillary arterioles were visualized by 2-photon-microscopy before and after betahistine application.
Key findings: There was no significant change in capillary diameter at sites of pericyte somas after betahistine application compared to controls, baseline or downstream controls, even though cochlear blood flow increased significantly. The two-photon measurements indicated an active dilation of precapillary arterioles.
Significance: Since we found no evidence that betahistine affects cochlear microcirculation by cochlear pericytes, its main mode of action is evidently active dilation of pre-capillary arterioles. These findings are in line with similar effects reported in the central nervous system and indicate an active effect on cochlear microcirculation.

1. Introduction

Menière's disease is clinically characterized by recurrent attacks of vertigo lasting minutes to hours, impaired hearing, tinnitus and fullness in the affected ear and was recently reclassified [1]. It is most likely caused by an endolymphatic hydrops [2,3], in which ruptures of the physical barriers of the endolymphatic space cause the recurring attacks of the aforementioned symptoms. Menière's disease is the second most common cause of otogenic vertigo [4].

Treatment options vary from dietary restrictions [5] to oral diuretics [6], intratympanic application of dexamethasone [7], lidocaine [8] or gentamycin [9]. In otherwise untreatable cases, surgery of the vestibular organ may be considered, even though the outcome is uncertain and associated with severe side effects [10]. Oral administration of betahistine hydrochloride, a histamine analogue, is a common treatment, in particular in central Europe [11]. It is generally considered beneficial in reducing the episodes of vertigo that are associated with Menière's disease, [11] although a randomized-controlled trial showed no benefit in the daily dosages examined [12].

Two modes of action have been proposed for the use of betahistine in vertigo and dizziness: Firstly, the inverse agonism of betahistine at the histaminergic H_3-receptor [13,14] is believed to aid the central nervous compensation in the vestibular nuclei in the case of a peripheral vestibular imbalance [15]. The second mode of action proposed is an active increase of cochlear microcirculation in the stria vascularis, [16–19] also mediated by its inverse agonism at the H_3-receptor and its interaction with the adrenergic α_2-receptor [14]. This is believed to lead to a reduction of the endolymphatic hydrops.

Studies dealing with the cochlear effects of betahistine have also shown systemic circulatory effects that match the changes in cochlear

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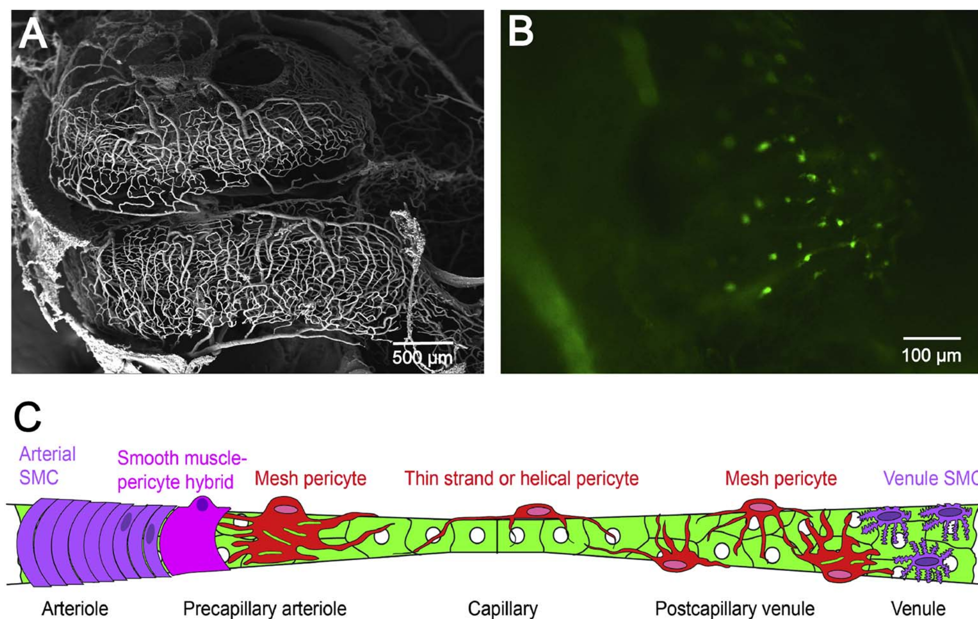

Fig. 1. A) Schematic display for pericyte heterogeneity, reproduced with permission from Hartmann et al. [45] B) Cast of the cochlear vascular network, reproduced with permission from Carraro et al. [46] C) In-vivo fluorescence microscopy of the stria vascularis with stained pericytes in a guinea pig.

microcirculation [14,16,18,19]. Hence, these observations gave rise to the question whether the increases in cochlear microcirculation are the results of a) an active regulation of cochlear blood flow or b) mere downstream effects.

In line with the argument of an active increase of cochlear blood flow is the fact that studies dealing with local regulation of blood flow in the cerebral cortex showed that the cerebral capillary pericytes take an active role in the regulation of capillary blood flow [20]. Pericytes are a heterogenous group of cells that adhere to the outer wall of capillaries (Fig. 1A, C). They play an important role in capillary stabilisation and regulation of local blood flow [21] and also seem to play an integral role in numerous central nervous diseases [22]. Since the capillary pericytes of the stria vascularis exhibit similar functional properties as the central nervous pericytes, such as the ability to contract and relax [23], and are of a similar embryological origin, a similar function of the capillary pericytes is probable. This view is further supported by the fact that exposition to tumor necrosis factor, a mediator in numerous inner ear pathologies, is capable of inducing active decreases in capillary diameters at sites of pericyte somas, while neutralisation of tumor necrosis factor causes a return to basal values [47]. A potential mode of action for betahistine is a direct effect on capillary pericytes, since pericytes have been known to increase local microcirculation upon H_1-stimulation in the retina [24] as well as expressing adrenergic receptors [25]. Additionally, some authors even consider the pre-capillary arterioles (Fig. 1A) to be a subpopulation of pericytes, [26,27] structures that have also been postulated to play a role in local regulation of cochlear blood flow [28].

In the light of these findings, we propose that if the effects of betahistine on cochlear microcirculation are specific to the cochlea, they will either be mediated by an active effect of betahistine on cochlear capillary pericytes, including the pre-capillary arterioles. Therefore, we investigated the effect of betahistine on a) cochlear capillary pericytes and b) pre-capillary arterioles.

2. Materials and methods

2.1. Ethics statement

All of the experiments in this study were reported to and approved by the responsible animal protection authorities ("Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg", Germany) under the license number 33.9-42502-04-12/0889.

2.2. Animals

The guinea pig is a well-established animal model for microcirculation [29] as well as Menière's disease [30]. The laboratory animals were guinea pigs from the Dunkin-Hartley strain bought directly from approved retailers (Envigo RMS GmbH, Rossdorf, Germany) weighing from 200 g to 450 g. Anesthesia was induced by intramuscular injection of a combination of ketamine (50 mg/kg bodyweight) and xylazine (5 mg/kg bodyweight) and sustained by repeated injections of half the dosage every 30 min.

2.3. Surgical approach

The surgical approach in order to visualize capillary cochlear microcirculation and capillary pericytes used in this experiment has been described repeatedly by this group [31–33] as well as others [34]. Thirty minutes prior to induction of anesthesia, an intraperitoneal dose of buprenorphine (0.5 mg/kg bodyweight) was given. After induction of anesthesia by the above mentioned protocol, the hair overlying the left lateral cervical region as well as the right periauricular area was removed. Following this, local anesthesia (lidocaine with epinephrine) was applied intracutaneously. Initially, an intravenous catheter was inserted into the left jugular vein, allowing i.v.-application of fluids as well as contrast material. After the implantation, the right external ear was removed and the bulla containing the cochlea was mechanically opened. After this, the periosteal vessels present in the bulla were removed above the cochlea. Then, an incision into the bony shell of the cochlea was carved above the second turn, using a no. 11 scalpel. The window carved measured approximately 500 × 500 µm. After the operation site had been rinsed with sterile saline solution, contrast material (fluorescein-labelled dextrane, molecular weight 500,000; 0.05–0.1 ml of a 5% solution in 0.9% NaCl; Sigma-Aldrich, Deisenhofen, Germany) was applied intravenously. In-vivo microscopy was then done by direct illumination with a Leica EL6000 light source (Leica Microsystems, Wetzlar, Germany) connected to a Leica M205 FA stereomicroscope (Leica Microsystems, Wetzlar, Germany). Once a portion of the stria vascularis had been visualized, pericytes were stained by topical application of a 5 mM of 4,5-diamofluorescein diacetate in dimethyl sulfoxide solution (Sigma-Aldrich, Deisenhofen, Germany) diluted 1:10 with sterile saline for 20 min. The images

obtained were processed by the Leica AS Software (Leica Microsystems, Wetzlar, Germany) and saved digitally for later analysis. An example of the images obtained by the fluorescence microscopy with stained pericytes can be found in the online supplemental material.

For the animals investigated by two-photon microscopy, the surgical approach was similar and has been described previously [28]. In short, the bulla was exposed surgically in the same manner as described above, but the bony exterior of the cochlea was left untouched. Then, fluorescein-labelled dextrane was injected intravenously and the guinea pig was fixated under a 2-photon microscope. The entire 2-photon setup utilized in the experiments was custom-made. It was mounted on a Newport RS 4000 sealed hole table top with tuned damping, stabilized by four Newport S-2000 high performance laminar flow stabilizers (Newport Spectra Physics GmbH, Darmstadt, Germany). Excitation was achieved with a Chameleon Vision II (Coherent Inc., Dieburg, Germany) titan-sapphire laser, working at 760 nm. Imaging was done through a custom-build 25 × magnification air objective.

The images were acquired using MATLAB (MathWorks, Natick, MA, USA). Since 2-photon-microscopy allows in-depth visualization of structures, images were saved in stacks of 40 pictures, similar to a tomography [28]. (Fig. 4B) The pictures were acquired in steps of 20 µm. The nature of the imaging process allowed three-dimensional reconstruction of individual vessels.

2.4. Treatment protocol

Twelve guinea pigs were randomly assigned to one of two groups treated with either betahistine (0.1 mg/kg bodyweight in 0.9% saline; Sigma-Aldrich, Deisenhofen, Germany) or placebo (0.9% saline). It has been established that the concentration used is the lowest possible concentration wielding the maximum effects on cochlear blood flow [18].

Once the setup was ready for recording, images of the stria vascularis were recorded for 90 s and digitally stored for subsequent analysis. Only those animals in which at least 8 pericytes were visible were eligible for further treatment. Then betahistine, or placebo, respectively, was infused intravenously. 7.5 min after the beginning of the infusion, images were acquired again. Following this, the animals were euthanized. The timepoint was chosen since the maximum effect on cochlear microcirculation was seen between 7 and 8 min after betahistine infusion in previous experiments [14,18,19].

For the two animals investigated by two-photon microscopy, the treatment protocol was the same. Images for later analysis were recorded prior to and 7.5 min after betahistine infusion.

2.5. Analysis

In the guinea pigs in which the cochlear pericytes were investigated, off-line image analysis was done by CapImage (Dr. Zeintl Engineering, Heidelberg, Germany), a software program that was designed for this purpose [35] and has repeatedly been used by this group [14,18,19,29,33,36,37]. Values obtained in these animals were vessel diameter at sites of pericyte somas, vessel diameter at a corresponding downstream control for each pericyte and the intravascular blood velocity at sites of pericyte somas. Cochlear blood flow in pl/s was calculated for each site by the formula postulated by Wayland: $q = (v/1.6) * (d/2)^2 * \pi$ [38]

Image analysis for the data obtained by two-photon microscopy was done by greyscale analysis in ImageJ (Build 1.51d for Mac, https://imagej.nih.gov/ij/).

For the pericytes, values are presented as relative change compared to baseline in order to correct for interindividual differences. Owing to the relatively small number of vessels investigated, the values for the pre-capillary arterioles are given as absolute values.

2.6. Statistics

In order to determine whether there were significant differences between the two groups, a linear mixed model was fitted (including a random effect for each guinea pig) by a restricted maximum likelihood approach. This was done by the software Project R (Build 3.2.5 for Windows, The R Project for Statistical Computing, http://www.r-project.org/).

To test for differences in the pre-capillar arterioles, a student's *t*-test for paired comparisons was done with the same software. A *p*-value of < 0.05 was considered significant.

3. Results

3.1. Effect of betahistine or placebo on capillary diameter at sites of cochlear pericytes and respective downstream controls

In both groups, capillary diameters at 152 pericyte sites and respective downstream controls were measured, of which 74 were treated with placebo and 78 were treated with betahistine. Betahistine infusion was followed by an increase in capillary diameters at pericyte sites of 0.031 ± 0.040 compared to baseline. (See Fig. 2) At downstream controls, a comparable increase of 0.030 ± 0.055 was observed. In comparison to that, treatment with placebo caused an increase of 0.005 ± 0.022 in capillary diameter at the site of pericyte somas. Similar values were quantified at downstream control sites, where the increase was 0.005 ± 0.022 as well.

The difference between pericyte sites and downstream controls as well as between betahistine and placebo was not statistically significant.

3.2. Effect of betahistine or placebo on blood velocity and cochlear blood flow

Blood velocity was quantified at each pericyte soma site. At sites treated with betahistine, blood velocity increased by 0.207 ± 0.182, while blood velocity at sites treated with placebo remained at 1.001 ± 0.046. This difference was statistically significant ($p < 0.001$, Fig. 3A)

Cochlear blood flow was then calculated for each pericyte soma site. Overall, the group treated with betahistine showed an increase in cochlear blood flow from baseline of 0.295 ± 0.166. In comparison, the group treated with placebo showed a smaller increase in cochlear blood flow of 0.012 ± 0.068. The fitted linear mixed model showed a significant difference in cochlear blood flow between betahistine and placebo ($p < 0.001$, Fig. 3B).

3.3. Effect of betahistine on pre-capillary arterioles

Overall, six precapillary arterioles were investigated by 2-photon microscopy. Before application of betahistine, the average diameter was

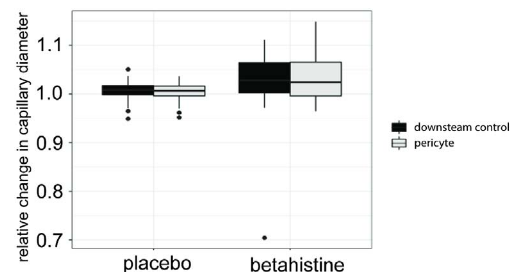

Fig. 2. Change of capillary diameter at sites of pericyte somas and downstream controls before and 7.5 min after betahistine or placebo infusion as measured by in-vivo fluorescence microscopy $n = 152$.

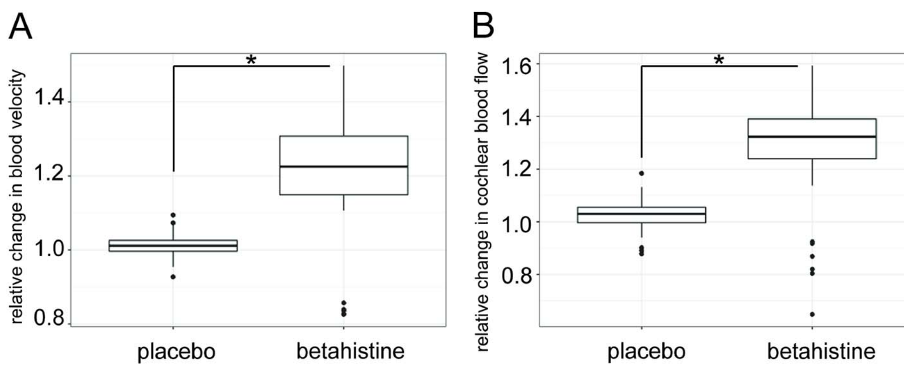

Fig. 3. A) Relative change of intravascular blood velocity at each pericyte site before and 7.5 min after infusion of betahistine or placebo determined by in-vivo fluorescence microscopy B) Relative change of intrasvascular blood flow at each pericyte site before and 7.5 min after infusion of betahistine or placebo determined by in-vivo fluorescence microscopy.
*$p < 0.05$ (linear mixed model) $n = 152$

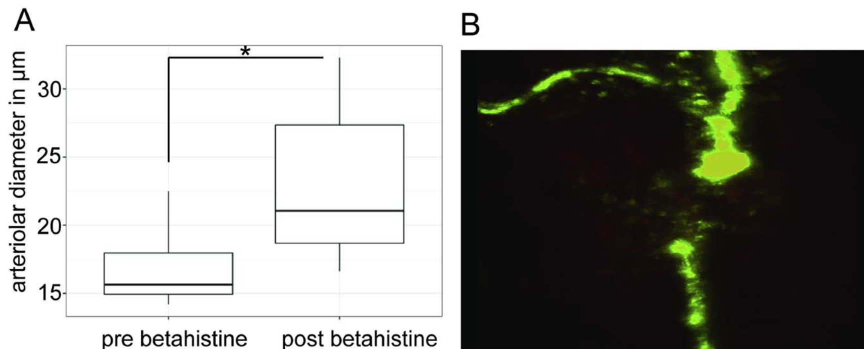

Fig. 4. A) Change of diameter of pre-capillary arterioles before and 7.5 min after betahistine infusion as measured by 2-photon microscopy B) Sample image of 2-photon microscopy of pre-capillary arterioles *$p < 0.05$ (*t*-test for paired comparisons) $n = 6$

16.899 ± 2.876 µm. After application of betahistine, the average artiolar diameter was 23.060 ± 5.721 µm. (Fig. 4) There was a significant ($p = 0.013$) change in arteriolar size. The increase compared to baseline was 0.358 ± 0.203.

4. Discussion

The present dataset shows that intravenous application of betahistine causes a significant increase in cochlear microcirculation which is in accordance with previous publications [14,16–19]. However, in the current study, we were not able to observe a significant effect on capillary diameter at sites of pericyte somas, which is in contrast to the effects and mode of action of other agents like tumor necrosis factor [47]. The fact that our study included only animals in which at least eight or more pericytes were visualized as well as the overall number of pericytes examined corroborate the validity of these results [23].

The fact that there were no effects on capillary diameter at sites of pericyte somas after betahistine infusion is probably caused by the great heterogeneity of the pericytes, namely due to functional differences between cochlear and cerebral pericytes. While cerebral pericytes are indeed able to contract and relax on neurotransmitter-stimuli [22], this is evidently not the case for cochlear pericytes [23]. Additionally, capillary pericytes have only been shown to be able to contract upon a pathological stimulus [47], thus suggesting that cochlear pericytes have, despite their numerous similarities to cerebral pericytes, an at least partially different function compared to cerebral pericytes.

However, other ways of actively regulating microcirculation apart from pericytes have been described in the cochlea, like the spiral modiolar artery [39] or pre-capillary arterioles of the spiral ligament. Taking into account the recent discussion about the role of pre-capillary arterioles and pericytes in the brain [20,22,26,27,40], an effect on the pre-capillary arterioles regulated by smooth muscle cells [26] seems probable. The data we acquired in the animals examined by two-photon microscopy showed a significant dilation in pre-capillary arterioles upon the infusion of betahistine, thus strongly supporting this hypothesis. Assuming that the increase of cochlear blood flow is caused by active dilations of pre-capillary structures, these findings would be in line with those made in the mouse brain [41].

There is currently a debate about the morphological and functional heterogeneity of pericytes. While some authors claim that contractions to regulate blood flow take place solely on a pre-capillary level, [27] other publications have claimed that contractions take place on a distinctly capillary level [20,23]. Nonetheless, our current data indicate that betahistine induces an increase in cochlear blood flow that is not actively mediated by the common capillary pericytes seen alongside the capillaries, but by the very distal arteriolar smooth muscle cells. Fittingly, these very distal cells are already considered pericytes by some authors [26].

Another aspect of cochlear microcirculation under betahistine treatment has to be addressed: whether the changes in cochlear blood flow are specific to the cochlea. Studies that have addressed betahistine effects on cochlear blood flow have repeatedly found changes in systemic mean arterial pressure that are congruent over the course of time with the changes in cochlear blood flow, [14,18,19] thus raising the question of whether the changes observed are actually downstream effects caused by changes in systemic arterial pressure. The fact that we were able to show active increases in the pre-capillary arterioles, thus suggesting active regulation, speaks against this argument. Similarly, betahistine has been reported to significantly increase blood flow in the cerebrum or the anterior inferior cerebellar artery, despite a significant drop in systemic mean arterial pressure [17,42]. This viewpoint is further supported by the fact that cochlear blood flow is subject to a strong autoregulation that would cancel out or prevent simple downstream effects [43].

Finally, the role of increases in cochlear microcirculation in MD needs to be addressed. While increases in cochlear microcirculation certainly contribute to the beneficial effects of betahistine in MD, it is likely that this is not the only mode of action: histaminergic H1-receptors have been described in the endolymphatic sac [44] and betahistine agonism at the H1-receptor might aid in the reduction of the endolymphatic hydrops. This highlights the need for further investigation regarding the effect of betahistine on the symptoms of Menière's disease in an animal model and the specificity of the effects observed on the pre-capillary arterioles and the stria vascularis,

respectively.

5. Conclusions

The data presented show that the increase of cochlear blood flow by betahistine is evidently not mediated by an active regulation of cochlear capillary pericytes but coincides with a dilation of pre-capillary arterioles, presumably by dilation of arteriolar smooth muscle cells.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2017.08.015.

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