PHARMACOKINETICS OF CAROVERINE AND ITS PROTECTIVE AND THERAPEUTIC ROLES IN NOISE-INDUCED HEARING LOSS FOLLOWING ROUND WINDOW ADMINISTRATION IN THE GUINEA PIG

ZHIQIANG CHEN

NATIONAL UNIVERSITY OF SINGAPORE

2003

PHARMACOKINETICS OF CAROVERINE AND ITS PROTECTIVE AND THERAPEUTIC ROLES IN NOISE-INDUCED HEARING LOSS FOLLOWING ROUND WINDOW ADMINISTRATION IN THE GUINEA PIG

ZHIQIANG CHEN
(Bachelor of Medicine)

A THESIS SUBMITTED
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF OTOLARYNGOLOGY
NATIONAL UNIVERSITY OF SINGAPORE

2003

ACKNOWLEDGEMENTS

This study was conducted through a joint program between the National University of Singapore and Karolinska Institutet, and was supported by the grants from the National Medical Research Council, Singapore, the Swedish Research Council, the Swedish Council for Working Life and Social Research, Karolinska Institutet, AMF Sjukförsäkringsaktiebolag, Stiftelsen Clas Groschinskys Minnesfond, Stiftelsen Lars Hiertas Minne, the Petrus, Augusta Hedlund Foundation and the Foundation Tysta Skolan. We thank Phafag AG, Schaanwald, Liechtenstein, for their supply of caroverine.

I wish to express my sincere gratitude to all who have contributed to this thesis and especially to:

Senior research scientist, Runsheng Ruan, my main supervisor, who introduced me to the world of science, for his guidance and support throughout my study.

Dr Maoli Duan, Stockholm, for his help in experiment design, for supervising electrophysiological experiments, detailed comments on manuscripts, and for his help in both science and life.

Associate professor Mats Ulfendahl, Stockholm, for giving me the opportunity to work in his lab and help in experiment design and detailed comments on manuscripts.

Associate professor Howsung Lee, for her support and supervising the HPLC experiment in her lab, Mrs. Yok Moi Khoo and Lu Fan for their HPLC technical assistance.

Senior research scientist, Deyun Wang, for his concern, encouragement and comments on my study.

Professor Erik Borg and Dr. Joseph Bruton for helpful comments on manuscript.

My friends in Singapore, Hongwei Ouyang, Qiang Liu, Jing Hao, Ruping Dai, Junfeng Ju, Sam and Zaw for their friendship and spending plenty of good time together.

My Chinese friends in Stockholm, Zhengqing Hu, Dongguang Wei, Guihua Liang, Jin Zou and Zhe Jin, for their friendship and help, and for discussion about everything.

The colleagues in the Center for hearing and communication research, Stockholm, Dr Leif Järlebark, Anette Fransson, Paula Mannström, Louise von Essen, Åsa Skjönsberg, and Sri for their friendship and help in both science and life.

TABLE OF CONTENTS

SUMMARY

Caroverine, an N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist together with antioxidant activity, has been shown to protect the inner ear from excitotoxicity and to be effective in the treatment of tinnitus, sudden hearing loss and speech discrimination disorders in presbyacusis. The clinical applications of most glutamate receptor antagonists are limited by the severe side effects when administrated systemically. Local application of caroverine directly onto the round window membrane (RWM) could be a more effective means and avoid side/adverse effect. For clinical application, basic information about the rate of drug diffusion across the RWM, systemic caroverine absorption, and elimination of drug from the inner ear is necessary. The first part of the thesis focused on the pharmacokinetics of caroverine in the perilymph, cerebrospinal fluid (CSF) and plasma after systemic and local applications at different dosages in guinea pigs. High-performance liquid chromatography was used to determine the drug concentrations. Our results show much higher caroverine concentrations in the perilymph with lower concentrations in CSF and plasma following local applications, as compared with systemic administration. Auditory brainstem responses (ABR) were measured to evaluate the changes in auditory function following local applications. The effects on hearing were transient and fully reversible 24 h after RWM applications. The findings suggest that local application of caroverine onto the RWM for the treatment of inner

ear diseases might be both safe and more efficacious while avoiding high blood and CSF caroverine levels seen with systemic administration.

The second and third parts of the thesis used the above RWM application model to test the protective and therapeutic effects of caroverine on noise-induced hearing loss in the guinea pig. The destruction of the afferent dendrite in the cochlea after noise exposure has been proved to be due to the excitotoxicity of excessive glutamate. Consequently, the production of reactive oxygen species plays an important role in cochlear damage. Caroverine was applied onto the RWM immediately prior to, 1 h or 24 h after noise exposure. The animals were exposed to 1/3 octave band noise centered at 6.3 kHz (110 dB, sound pressure level, SPL) for 1 h and the ABR was measured before and at regular time intervals after noise exposure. Our results show that caroverine can significantly protect the auditory function against noise trauma when applied immediately prior to noise exposure. The hearing was significantly rescued by caroverine when administrated 1 h, but not 24 h, after noise trauma. The two parts of the thesis demonstrated that caroverine could significantly protect and rescue the auditory function against noise trauma when applied prior to or 1 h after noise exposure. Thus, pharmacological protection of the cochlea against noise is possible and may be of great clinical potential.

ABBREVIATIONS

ABR	auditory brainstem response
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CSF	cerebrospinal fluid
dB	decibel
HD	high dose
HPLC	high-performance liquid chromatography
IHC	inner hair cell
IV	intravenous
LD	low dose
NIHL	noise-induced hearing loss
NMDA	N-methyl-D-aspartate
OHC	outer hair cell
PTS	permanent threshold shift
ROS	reactive oxygen species
RWM	round window membrane
SPL	sound pressure level

INTRODUCTION

Mammalian auditory anatomy

The mammalian auditory system consists of the outer, middle and inner ear with the auditory nerve and the central auditory pathway (Fig. 1). The outer ear is composed of the auricle and the external auditory canal. The middle ear includes the tympanic membrane, the ossicles with the associated muscles, tendons, ligaments, and the Eustachian tube. The three ossicles are the malleus, incus and stapes. The tensor tympani is attached to the malleus and is innervated by the trigeminal cranial nerve. The stapedium muscle is attached to the stapes, and is innervated by the facial cranial nerve.

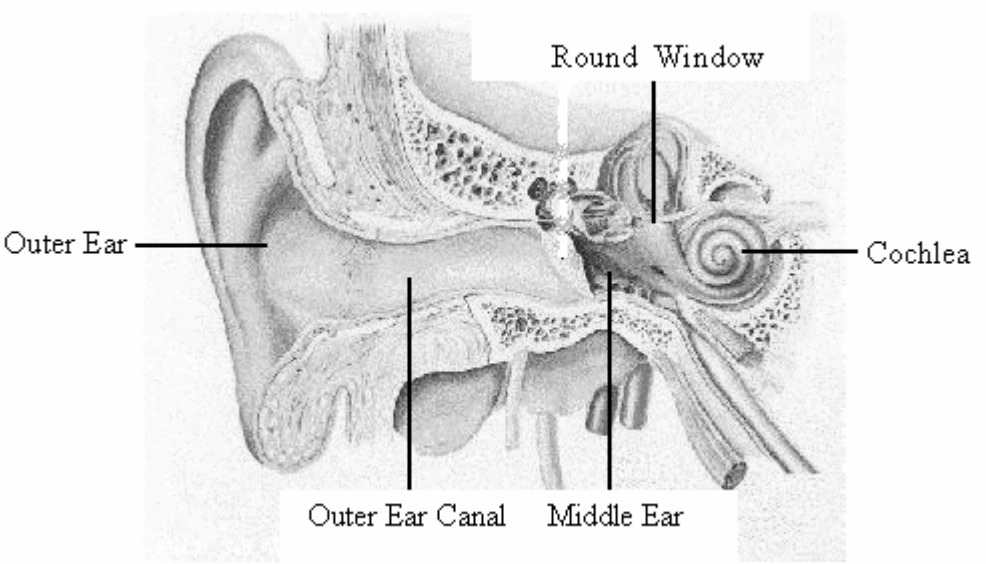


Fig.1. The structure of the human ear. The auditory system includes outer ear, middle ear, inner ear, auditory nerve and central auditory pathway. The hearing organ is in the inner ear and called cochlea. The round window is the only opening covered with membrane which separates the scala tympani from the round window niche. Modified from Alec N. Salt, Washington University, 2003.

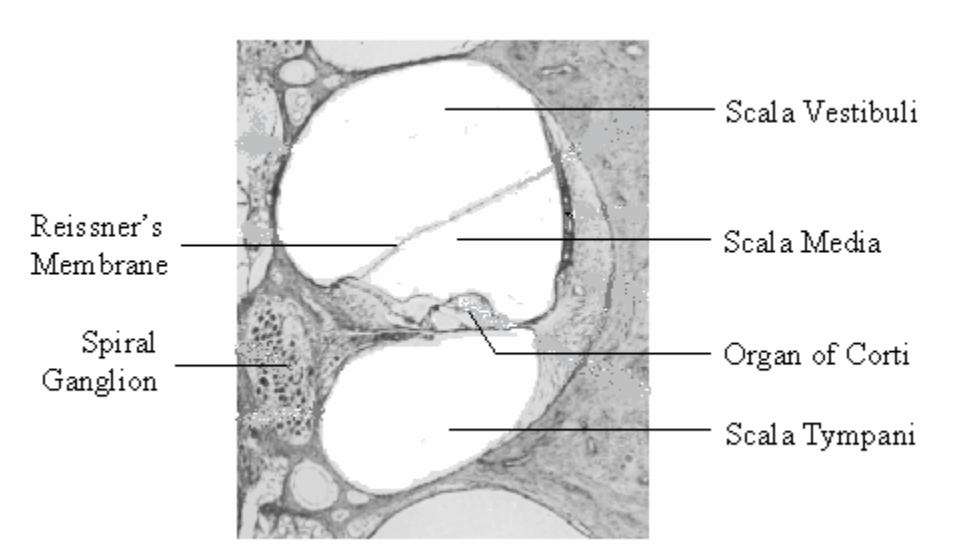


Fig. 2. The structure of the cochlea. Reissner's membrane and the basilar membrane separate the cochlea into three compartments. The scala tympani and scala vestibuli are filled with the perilymph which is similar to the extracellular solution with high sodium and low potassium. The scala media is filled with endolymph which is similar to intracellular solution with high potassium and low sodium. The organ of Corti is situated on the basilar membrane and osseous spiral lamina. Modified from Alec N. Salt, Washington University, 2003.

The inner ear is deeply embedded in the temporal bone and includes the hearing and vestibular organs. On the outside the hearing organ resembles a snail shell and is called the cochlea. The middle ear and inner ear communicate via two openings in the temporal bone, the oval and round windows. The innermost middle ear ossicle, the stapes, is inserted in the oval window, and a flexible membrane covers the round window. On the inside, the cochlea is divided into three compartments, scala vestibuli, scala media, and scala tympani (Fig. 2). The scala media is separated from the scala vestibuli above by Reissner's membrane and from the scala tympani below by the basilar membrane. On the innermost aspect, the basilar membrane goes from the spiral lamina in the modiolus to the outermost spiral ligament and

stria vascularis. In the apical part of the cochlea the two outer scalae, the scala vestibuli and scala tympani, are joined together via the helicotrema and are filled by the perilymph. The scala media, the compartment between the scala vestibuli and tympani, is filled by endolymph.

The organ of Corti is situated in the scala media on the basilar membrane and osseous spiral lamina. In human the basilar membrane is approximately 0.12 mm wide at the base and increases to approximately 0.5 mm at the apex. The major components of the organ of Corti are one row of inner hair cells (IHCs), three rows of outer hair cells (OHCs), supporting cells (Deiters, Hensen, Claudius), tectorial membrane, and the reticular lamina-cuticular plate complex (Fig. 3). Supporting cells provide structural and metabolic support for the organ of Corti. Inner and outer hair cells are important in transduction of acoustic energy into neural energy. There are approximately 3,500 IHCs and 12,000 OHCs in each cochlea in human (Ulehlova et al., 1987). A sensory bundle containing three rows of stereocilia, which on the IHC form a shallow U-shape and on the OHC a W- or V-shape crowns the apical surfaces of both types of hair cells. The OHC stereocilia are firmly attached to the underside of the tectorial membrane, while the IHC stereocilia are either freestanding or only delicately attached to the membrane (Lim, 1980). The tight junctions with the reticular lamina seal the apices of the hair cells. The basilar membrane is permeable to ions, and consequently the bodies of the hair cells are surrounded by the perilymph. In contrast, the apical faces of hair cells with their stereocilia and the entire reticular lamina are bathed by the endolymph. The

IHCs are the primary sensory cells that transit information to the brain, while the function of the OHC is perceived as that of a cochlear amplifier that refines the sensitivity and frequency selectivity of the mechanical vibrations of the cochlea. The positive feedback force provided by OHCs cancels the viscous and dissipative forces exerted by the surrounding fluid and other cells, and leads to a 100-fold increase in the sensitivity of the cochlea by enhancing resonance responses along the partition (Robles and Ruggero, 2001).

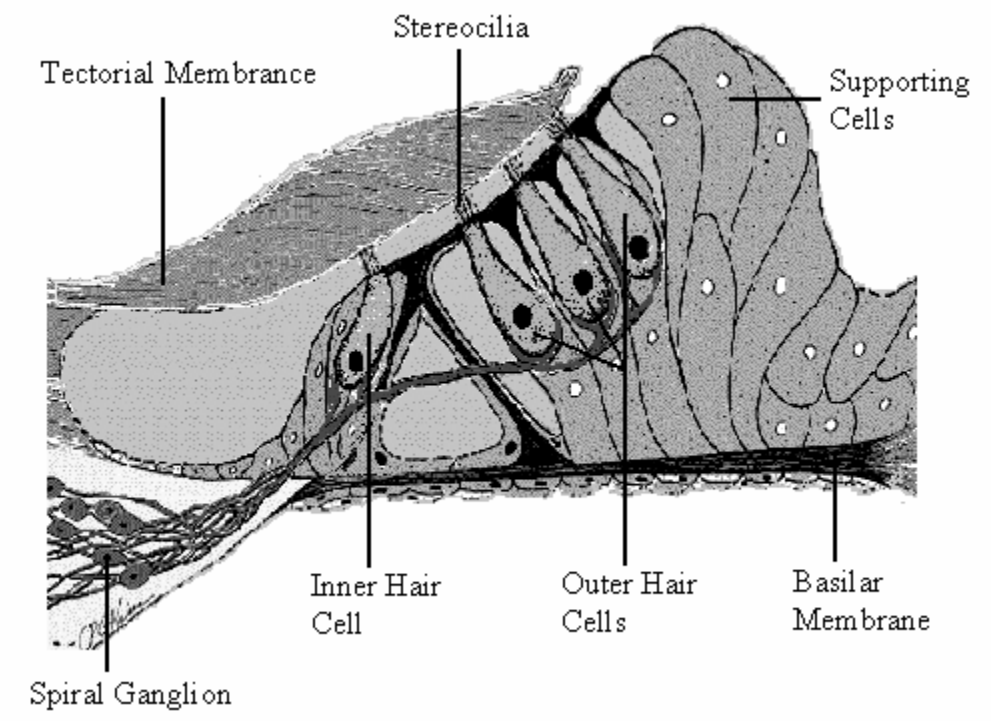


Fig. 3. The structure of the Organ of Corti. The Organ of Corti consists of one row of inner hair cells, three row of outer hair cells, supporting cells, tectorial membrane, and basilar membrane. There are stereocilia on the top of hair cells. Modified from Atlantic coast ear specialists, PC, 2003.

The hair cells of the cochlea are innervated by both afferent and efferent neurons. The afferent neurons carry sensory information from the hair cells to the central nervous system. About 90-95 % of the afferent nerves come from the IHCs. Each IHC receives about 20 fibers, whereas each of the afferents to the OHCs innervates about 10 OHCs at the base and 50 at the apex of the cochlea (Spoendlin, 1972). The efferent neurons descend from the superior olivary complex in the brainstem to the cochlea. Unmyelinated efferents originate from the lateral superior olivary nucleus, descend mostly ipsilaterally, and terminate on the afferent dendrites of the IHCs (Warr and Guinan, 1979). Myelinated fibers from the medial superior olive go mostly contralaterally toward the basal part of the OHCs.

Blood-labyrinthine barrier

The two inner ear fluids, the endolymph and perilymph (Fig. 4), are essential to both hearing and equilibration. The sensory cells are bathed with endolymph at their apical ciliated surfaces and with perilymph at their basal synaptic ones. The two fluids differ dramatically in composition: the endolymph is a positively polarized solution of potassium salts that is similar to intracellular fluid, whereas the perilymph has a chemical composition resembling that of a plasma ultrafiltrate (Sterkers et al., 1988).

Three different theories of the production and turnover of endolymph are proposed: the longitudinal, radial, and dynamic theories. According to the longitudinal theory, endolymph is produced by the secretory epithelia of the cochlea and the vestibule

and is reabsorbed in the endolymphatic sac (Guild, 1977). The radial theory suggests that endolymph is produced and reabsorbed locally (Naftalin and Harrison, 1958; Lawrence et al., 1961). That is, endolymph is secreted by the stria vascularis and the Reissner's membrane acts as a filter through which fluids and electrolytes pass from endolymph to perilymph. The dynamic theory incorporates both the longitudinal and the radial theories (Lundquist, 1976). Longitudinal flow is considered important for the transport and reabsorption of cellular debris and high molecular waste products via the endolymphatic sac, while radial flow is believed important for ion exchange to maintain the characteristic electrochemical composition of endolymph as well as the endocochlear electric potential.

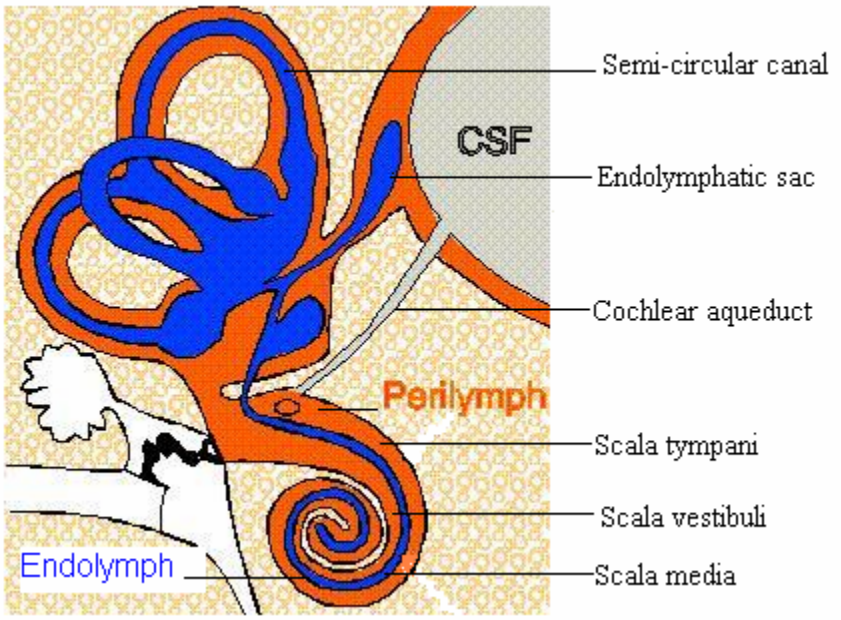


Fig. 4. The inner ear fluid compartments. The endolymph is proposed to be produced by the secretory epithelia of the cochlea and vestibule and resbsorbed in the endolymphatic sac, or be secreted and reabsorbed by the stria vascularis, separately or in combination. The perilymph is thought to come from CSF and/or blood vessels. Modified from Alec N. Salt, Washington University, 2003.

The perilymph seems to have three potential origins, alone or in combination (Medina and Drescher, 1981; Manzo et al., 1990; Thalmann et al., 1992). One source is the cerebrospinal fluid (CSF), which reaches and mixes with the perilymph of the scala tympani via the cochlear aqueduct. The cochlear aqueduct maintains its relatively patency in lower-order mammals, whereas in human it has a more rudimentary structure. The second origin of the perilymph is the CSF that enters the cochlea through perivascular spaces and vestibulocochlear nerve sheaths at the distal end of the internal auditory canal. The third and probably the major source is from the blood vessels that supply the inner ear itself. It is suggested that the origin of scala tympani perilymph is different from that of scala vestibuli perilymph (Sterkers et al., 1988). Following intravenous administration of the radioactive-labeled hydrophilic molecules mannitol and sucrose in animals, these molecules appeared faster and reached higher concentration in the scala vestibuli than in the scala tympani or CSF. However, another study showed that no significant differences in the average concentrations of seven-selected biochemical substances within the perilymph following cochlear aqueduct occlusion (Scheibe and Haupt, 1985). The question of whether the cochlear aqueduct provides a physiological biochemical communication between the CSF and perilymph in human is still under debate and remains controversial. Consequently, any study designed to assess pharmacokinetics profiles of chemicals in the inner ear fluids should also include similar profiles of the CSF.

Permeability of round window membrane

The round window membrane (RWM) is located in medial wall of the middle ear, within the round window niche (Fig. 5). The round window niche, which is posteroinferior to the promontory, has a triangular shape and is bound medially by the RWM (Goycoolea et al., 1990). There are commonly folds of middle ear mucosa, which is termed false round window membrane, at the entrance of the niche. The RWM separates the niche from the scala tympani and its outer surface is directly inferiorly. The cochlear aqueduct, which connects the perilymphatic space with the cerebrospinal space, is located close to the posterior part of the RWM. The oval window is directly superior to the RWM.

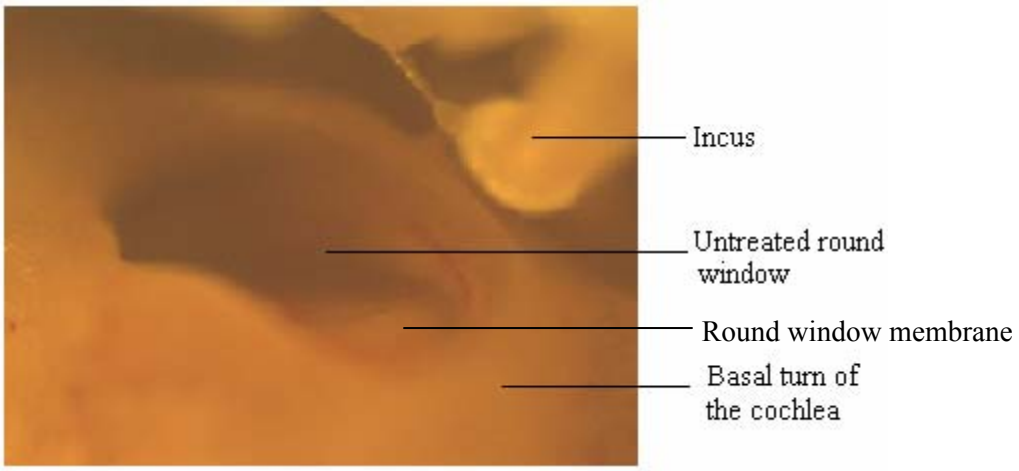


Fig. 5. The round window membrane (RWM). The RWM bounds the round window niche and separates the niche from the scala tympani.

The RWM is thicker at the edges and has a slight convexity towards the scala tympani (Carpenter et al., 1989). The average thickness in human is 70 μm and does not change with age. The membrane consists of three layers: an outer epithelium, a middle connective tissue, and an inner epithelium (Fig. 6) (Goycoolea 2001). The outer epithelium consists of a single layer of cells continuous with the mucous membrane lining the middle ear. The middle connective tissue contains fibroblasts, collagen, elastic fibers, and blood and lymph vessels. It is the dominating part of the RWM and is thought to be in conjunction with the mucoperiosteum of the otic capsule. The inner epithelial cells are squamous and consist of several layers of thin cells, which are continuous with the mesothelial cells of the scala tympani. The extracellular spaces are large and no basal lamina separates this layer from the middle fibrous layer.

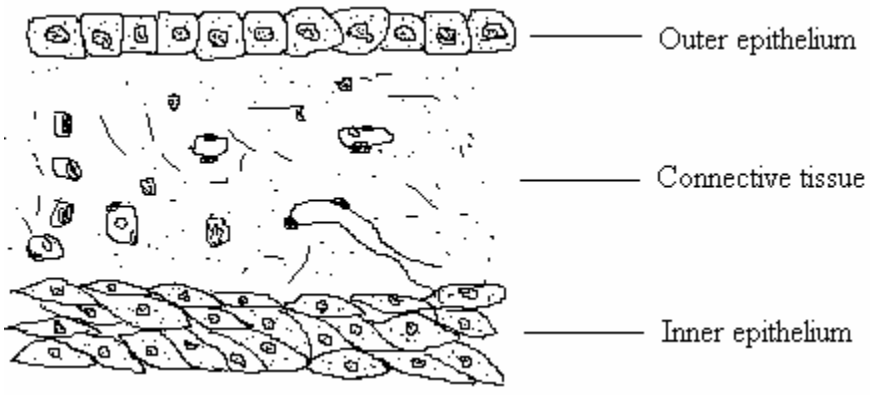


Fig. 6. Schematic drawing of the round window membrane. The RWM consists an outer single-layer epithelium, a middle connective tissue, and an inner stratified squamous epithelium.

The function of the RWM is presumed to release mechanical energy and/or conduct sound to the scala tympani (Scarpa A, 1962). Based on experimental studies and anatomical observations, the RWM may also act as a barrier to ototoxic substances in the middle ear and participate in the secretion and absorption of substances (Richardson et al., 1971; Miriszlai et al., 1978). Animal experiments show that the RWM behaves like a semipermeable membrane. Many substances with both low and high molecular weights have been demonstrated to penetrate through the RWM when placed in the round window niche (Goycoolea and Lundman, 1997; Goycoolea 2001). These substances include sodium ions, antibiotics, antiseptics, arachidonic acid metabolites, local anesthetics, toxins and albumin. Tracer studies using cationic ferritin, horseradish peroxidase, 1 μm latex sphere and neomycin gold spheres have shown the permeability of the RWM to these substances when applied in the middle ear side in chinchillas, guinea pigs, cats, Mongolian gerbils, and rhesus monkeys. The permeability of the RWM can be influenced by the factors such as size, configuration, concentration, liposolubility and electrical charge of the substance, and the thickness and the condition of the RWM (Goycoolea et al., 1988). The substances placed on the RWM may traverse through the cytoplasm as pinocytotic vesicles or through different channels in between cells in the epithelium. In the connective tissue layer, cells can phagocytize the substance and traverse towards perilymph and/or penetrate blood or lymph vessels in this layer (Goycoolea and Lundman, 1997). Theoretically, after the substance reaches the perilymph it would go towards the CSF through the cochlear aqueduct, up to the scala tymphi, or find way to the endolymph.

Local RWM application for the treatment of inner ear disorders

Clinically, there is increasing interest in the local delivery of drugs directly into the inner ear across the intact RWM. The main advantage of the local method is that the drug will bypass the blood-labyrinth barrier and directly enter the inner ear, resulting in higher inner ear concentration and reduced systemic absorption and toxicity. In cases of Ménière's disease, the instillation of gentamicin or streptomycin solutions into the middle ear has been widely used as a method of suppressing vestibular function in the affected ear (Blackley 1997). This approach avoids the risk of damaging the non-affected ear, as would occur with systemic treatments. Experimental studies are developing uses for a wide variety of agents, including steroids, local anesthetics, antioxidants, glutamate receptor antagonists, neurotrophins and vectors for gene therapy, delivered on or through the RWM, as treatments for various inner ear disorders (Coles et al., 1992; Kopke et al., 1996; Blackley 1997; Seidman 1998; Stover et al., 1999; Yage et al., 1999). Furthermore, several specific delivery systems have been developed for more controlled local applications, including round window microcatheter (Durect Inc., Cupertino, CA; IntraEar, Inc., Denver, CO), the MicroWick inserted through a tympanic membrane vent tube into the round window niche (Silverstein, 1999), and a bone-anchored, totally implantable drug delivery system (TI-DDS) composed of a micropump, a drug reservoir and a septum port (Lehner et al., 1997).

However, most drug application protocols are empirically based because of the unknown pharmacokinetics of the drugs in the inner ear. The amount and distribution of applied substances within the inner ear is poorly understood due to the considerable technical difficulties in making such measurements. As a result, the consequences of changes in delivery method, applied drug concentration, or even small alterations in treatment protocols have been difficult to predict. For instance, gentamicin has been applied onto the RWM by single or repeated intratympanic injection, by application onto the gelfoam placed on the RWM, by applying onto a wick, or by continuous delivery via implanted catheters. The therapeutic results varied significantly among these approaches (Plontke et al., 2002). The variation among different groups may be attributable to both different dosing regimens and application methods, although a correlation of outcome to both dosage and application method has yet to be established. The variability in results and the lack of uniformity in treatment protocols make it important to investigate the distribution and elimination of the drugs in the cochlea fluid spaces and the influence of different methods of application.

Neurotransmission in the cochlea

Afferent system

The mechanical stimulation results in the release of neurotransmitter from the inner hair cells to afferent nerve (Fig. 4). There is abundant evidence that glutamate is the most likely neurotransmitter at the synapse between the IHC and its afferent neuron

(Klinke, 1986; Altschuler et al., 1989; Eybalin and Pujol, 1989; Felix and Ehrenberger, 1990; Eybalin, 1993; Puel, 1995; Ruel et al., 1999; Glowatzki and Fuchs, 2002). Electrophysiological studies showed that glutamate and aspartate increased the spontaneous firing in the primary auditory neurons when applied to the scala tympani (Bobbin, 1979). By using microiontophoretic technique, glutamate was demonstrated to increase the afferent neuron firing rates when applied in the vicinity of the synapse (Ehrenberger and Felix, 1991). The glutamate-induced activity was blocked by glutamate competitive and non-competitive antagonists (Cousillas et al., 1988; Ehrenberger and Felix, 1991; Devau et al., 1993). Immunohistochemical studies have demonstrated that a selective immunoreactive staining for glutamate in the IHCs as well as spiral ganglion neurons (Altschuler et al., 1989; Usami et al., 1995).

There are two main classes of glutamate receptors in the cochlea: the ion channel linked (ionotropic) receptors responsible for the rapid neuronal excitation, and the metabotropic receptors coupled via G-proteins to intracellular messengers to mediate relatively slow glutamate responses. The ionotropic glutamate receptors are predominately located post-synaptically (Petralia and Wenthold 1995) and are divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Fig. 7) (Ryan et al., 1991; Niedzielski and Wenthold, 1995; Usami et al., 1995; Matsubara et al., 1996). AMPA receptor is also found to locate pre-synaptically on the hair cells, probably providing a negative feedback to the response of neurotransmitter release

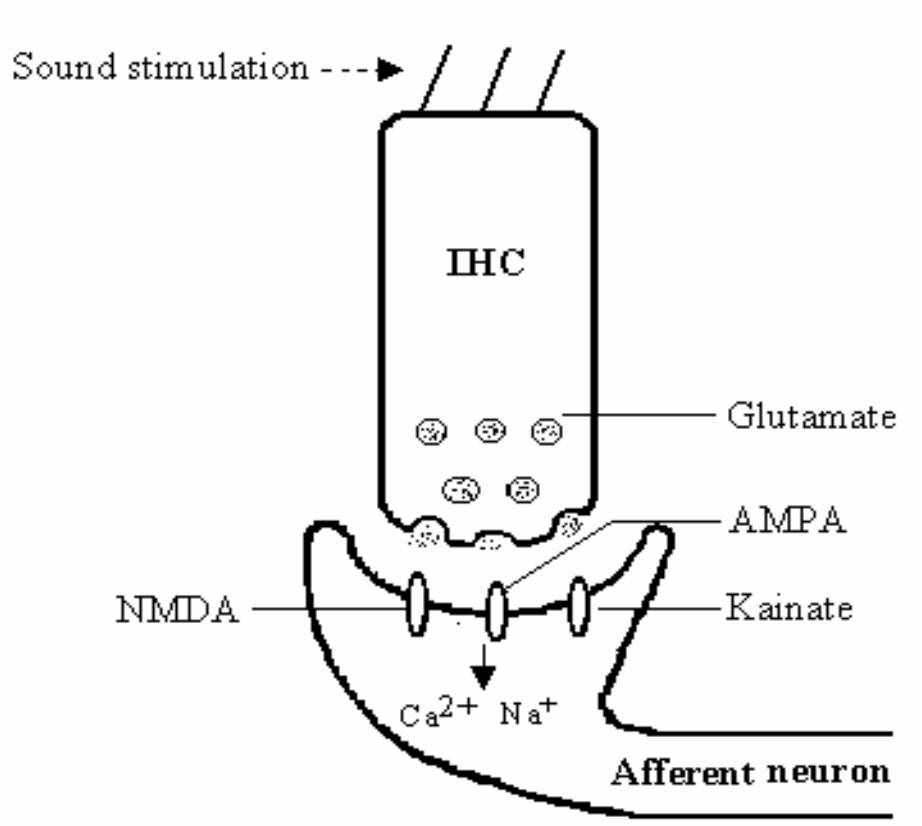


Fig. 7. The afferent neurotransmission. It is generally accepted that glutamate is the major neurotransmitter between the inner hair cell and its afferent neuron. There are three types of ionotropic glutamate receptors on the neurons: AMPA, NMDA and kainate receptors. Physiologically, the sound is transmitted from outer ear, middle ear to the inner ear and stimulates the inner hair cells. Under stimulation, the inner hair cells will release glutamate to the synapse and the glutamate will bind to its receptors and cause the influx of ions into the neurons. The influx the ions will depolarize the neuron and initiate action potential. This signal will be transmitted via the auditory nerve to the brain and perceived as sound.

(Matsubara et al., 1996). AMPA receptors are activated at low-to-moderate sound stimulus, whereas NMDA receptors are activated by high-intensity sounds (Felix and Ehrenberger, 1991; Puel et al., 1991). The role of NMDA receptors remains controversial. For instance, iontophoretic application of NMDA induced excitation of the primary auditory nerve fibers (Felix and Ehrenberger, 1990), but no effect of NMDA has been found on isolated primary auditory nerve soma or in intact

preparation (Ruel et al., 1999, 2000). In isolated primary auditory nerve soma, AMPA induced a fast onset and rapidly desensitized inward current, while kainate initiated only a nondesensitizing, steady-state current (Nakagawa et al., 1991; Ruel et al., 1999). Recently, GYKI 53784 has been demonstrated to be one of the most selective antagonists for AMPA receptors (Bleakman et al., 1996). Perfusion of 10 μM GYKI 53784 significantly reduced the spontaneous discharge rate of the auditory nerve fiber. The activity of the fiber was completely abolished by 50 μM GYKI 53784, suggesting that AMPA receptors, not kainate or NMDA receptors, predominately mediate the fast excitatory transmission at the IHC-afferent nerve synapse (Ruel et al., 1999, 2000).

Supporting cells take up excessive glutamate released from the presynaptic body in a Na^+-dependent manner through the glutamate transporter (GLAST) (Gulley et al., 1979; Eybalin and Pujol, 1983; Li et al., 1994; Furness and Lehre, 1997; Rebillard et al., 2003). GLAST is enriched in those membrane domains that face the synaptic region. Glutamate is converted to glutamine by glutamine synthetase and transferred to hair cells by unknown mechanisms. In the hair cells the glutamine is converted to glutamate by phosphate-activated glutaminase and glutamate is then accumulated in vesicles and ready for a new round of exocytosis.

Efferent system

According to the site of origin in the brain stem, the efferent supply to the cochlea is divided into the lateral efferent and the medial efferent innervations. The lateral

efferent system coming from the lateral superior olive modulates the activity of the auditory nerve dendrites located beneath the IHCs. Biochemical, pharmacological and immunochemical experiments have demonstrated that the lateral efferent system may use acetylcholine (ACh), gamma aminobutyric acid (GABA), dopamine and several neuropeptides such as enkephalin and calcitonin gene-related peptide (CGRP) as neurotransmitters (Eybalin, 1993). ACh is thought to be one important efferent neurotransmitter since Schuknecht et al. (1959) reported that the deefferented cochlea showed negative stain for acetylcholinesterase in contrast to the intact cochlea. ACh increases the spontaneous and glutamate-mediated firing activity in the afferent fibers, whereas GABA reduces glutamate-induced depolarization and has little effect on spontaneous activity (Felix and Ehrenberger, 1992). Dopamine, another efferent neurotransmitter, reduces the cochlear potentials only at the highest intensities of sound stimulation (d'Aldin et al., 1995; Ruel et al., 2001).

The medial efferent system, originating from medial nuclei of the superior olivary complex, modulates the activity of the OHC. There are numerous reports indicating that ACh is the main neurotransmitter in the medial efferent system, while the two other neuroactive substances, GABA and CGRP, may play some role (Puel, 1995). When the medial olivery complex bundle was stimulated, ACh increased in the cochlea (Norris and Guth, 1974). Kujawa et al. (1992) showed that ACh, when applied directly in the cochlea, decreased the amplitude of the DPOAEs, and this can be prevented by inhibitors of ACh such as curare and strychnine. Furthermore,

it has been revealed that ACh is the neurotransmitter mainly in the basal turns (Eybalin and Pujol, 1987), whereas GABA may be involved in the apical part (Eybalin et al., 1988). There are two major ACh receptors represented on the OHCs. Muscarinic receptors are preferentially activated by muscarine that mediates depolarization and facilitation of the afferent firing. Nicotinic receptors, with α9 and α10 units as its main component, are excited by nicotine and mediate hyperpolarization and suppression of afferent firing (Elgoyhen et al., 1994; Glowatzki et al., 1995; Vetter et al., 1995; Elgoyhen et al., 2001; Weisstaub et al., 2002). ACh induces an outward K+ current by binding to the nicotinic receptors, resulting in OHC hyperpolarization (Housley and Ashmore, 1991) and, subsequently an increase in the CM (Bobbin and Konishi, 1971). Apart from the activation of K^+ current, nicotinic receptors are supposed to be involved in the modulation of cell motility. In the isolated OHCs, inositol 1,4,5-trisphosphate induced motile responses (Schacht and Zenner, 1987).

Transduction of sound

Sound of different frequencies is transferred from the outer ear canal to the tympanic membrane. The pressure in the middle ear is increased from the tympanic membrane with its larger area to the oval window with its smaller area. The vibration of the stapes on the oval window produces a pressure difference in the scala tympani and scala vestibuli. The sound wave will displace the basilar membrane. The displacement pattern of the basilar membrane is a traveling wave. Because the basilar membrane is stiffer at the base than in the apex and the stiffness

component is distributed continuously, the traveling wave always progress from base to apex. The principal mechanical basis for cochlear frequency analysis was first demonstrated by Georg von Békésy (1960) by using cadaver cochleae from human, for which he was awarded the 1961 Nobel Prize for Physiology or Medicine. The peak or maximum amplitude of basilar membrane displacement varies as a function of stimulus frequency. Traveling waves produced by high-frequency sounds have maximum displacement near the base of the cochlea, whereas the waves to low-frequency sounds have the maximum toward the apical region. Traveling wave to high-frequency sounds does not reach the apical region of the cochlea, but wave to low-frequency sounds can travel the entire length of the basilar membrane. The mechanism for the sharply tuned peak in the mechanical traveling wave involves activity of the OHCs that enhances the motion of the basilar membrane at frequencies near the best frequency of the particular cochlear location. Factors contributing to the enhancement, also called the cochlear amplifier, may include the motility of OHCs and the mechanical properties of the stereocilia and tectorial membrane.

The movement of the basilar membrane causes a shearing motion between the stereocilia and the tectorial membrane. The tip of the stereocilia contains the cationic channel (Denk et al., 1995). The resulting deflection or sliding of the stereocilia alters the opening probability of mechanically sensitive ion channels. The flow of potassium ions into the sensory cell is modulated by the opening and closing of ion channels of the stereocilia. Stereocilia displacement in one direction

makes cation-selective channels near the tips of the stereocilia open, and the endolyphatic potassium ions enter the hair cells and produce depolarization. The resulting intracellular depolarization leads to the activation of voltage-sensitive calcium channels. The calcium inflow releases the neurotransmitter into postsynaptic terminals and causes the activation of the afferent nerve fibers. The mechanical sense is then transmitted to the central nervous system (Avraham, 1997). Deflection of stereocilia in the other direction decreases the open probability of the ion channel and leads to hyperpolarization (Flock, 1965; Hudspeth, 1983).

Auditory brainstem response

The auditory brainstem response (ABR) is by far the most widely used of the various auditory evoked potentials in both the clinic and experimental study. The pioneering work of Berger (1929) revealed that it was possible to record the electrical activity of the brain (electroencephalogram, EEG) from the electrodes placed on the human scalp. A change in EEG occurs when a stimulus is presented. Davis et al. (1939) first described the auditory evoked potential obtained from alert and sleeping human beings. They found small but consistent changes in raw EEG tracings when a repeatable auditory stimulus was introduced. Later, Clark et al. (1958, 1961) made great contributions to extract tiny evoked potential responses from noise background by developing the principle of algebraic summation of bioelectric events. It was Jewett and his colleagues (1970, 1971) that defined the ABR waves and identified the origin of the far-field scalp-recorded ABR. Generally, the ABR has five characteristic waves, wave I-V (Fig. 8). It was revealed

that wave I was the activity from the eighth nerve; wave II from the cochlear nucleus; wave III from the superior olivary complex; wave IV from the nucleus of the lateral lemniscus; and wave V from the inferior colliculus. Since then, the ABR has become a useful tool for the audiologist, otologist, and the neurologist.

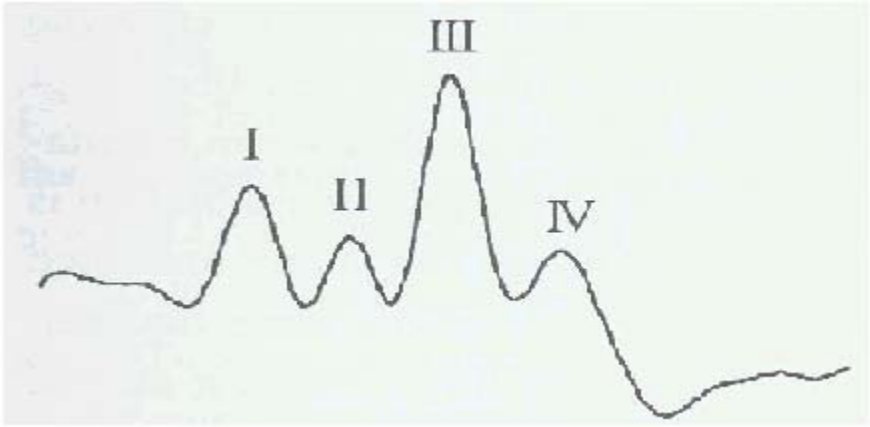


Fig. 8. A typical ABR waveform of the guinea pig. Wave I is the activity from the eighth nerve which innervates the cochlea; wave II from the cochlear nucleus; wave III from the superior olivary complex; wave IV from the nucleus of the lateral lemniscus and inferior colliculus.

Practically, the ABR is obtained from two electrodes placed on the skull surface with the use of acoustic stimuli. The click stimuli are most commonly used for generating the ABR waves, while tone bursts are also used for various applications. The reason for using transient stimuli like clicks is that many neurons must be made to fire at essentially the same time (synchronously) in order to elicit a measurable action potential. With the characteristics of abrupt onsets, short durations and broad spectra, clicks activate a large number of hair cells along the basal part of the cochlea, where the speed of the traveling wave is very fast. This, in turn, causes

essentially simultaneous firing of the auditory nerve fibers associated with these basal turn hair cells. The time domain of the ABR recording is within 10 ms after acoustic stimulation. The rise time of the stimuli is important to the ABR wave and should be 2 ms or less. The stimulus repetition rates are between 10 to 20 per second. Bipolar electrodes are used for ABR recording, with positive electrode on the vertex and negative behind the ear. The filter and amplifier play critical roles for a well-defined ABR wave. The filter is commonly 0.1-3.0 kHz. The amplifier produces the amplification to 100,000 times. The final stage for obtaining an ABR waveform is the averaging of the response, which improves the signal to noise ratio. The resultant waveform consisting of a series of waves can then be analyzed for latency and amplitude. Latency is the amount of time that has elapsed since the stimulus was presented. Latency is a more sensitive measurement than amplitude and is used in most clinical and experimental studies to determine the place of the hearing loss. The shift of latencies of early and late waves in parallel would be consistent with mainly a cochlear effect whereas prolongation of later waves relative to wave I would be indicative of contributions from central pathways in addition to the cochlea.

The ABR has been widely used for the evaluation and diagnosis of the peripheral auditory system and related pathology, for the integrity of the acoustic nerve and caudal levels of the brainstem pathway (Hecox and Jacobson, 1984). In particular, the ABR is used to estimate the hearing for infants and patients who cannot be tested using routine behavioral audiologic procedures.

Noise-induced hearing loss

Studies on the pathology of the noise-induced damage to the cochlea started more than one century ago (Toynbee, 1860). Habermann (1890) first demonstrated that noise destroys the nerves and the hair cells in human inner ears by light microscopy. Since then, intensive studies have been performed on animal ears as well as human temporal bones by the introductions of surface-specimen technique, scanning electron microscope, transmission electron microscope, etc. Intense sound stimulation results in various structural changes leading to functional auditory damage. The organ of Corti is the weakest and most susceptible to damage, while the inner ear impairment is by far the main cause of hearing loss. The pattern and the time course of damage within the cochlea are two important factors. Intense noise may cause impairments to the stereocilia, hair cell soma and afferent dendrites (Spoendlin, 1971; Robertson, 1983). The classical pattern of hair cell degeneration starts with OHCs from the first row, then the IHCs and subsequently OHCs from the second and third rows. Fredelius et al. exposed guinea pigs to intense continuous noise and examined histologic and ultrastructural changes in maximal injury area and the surrounding border zones within the cochlea from 5 min to 4 weeks following noise exposure (Fredelius, et al., 1988; Fredelius, 1988). Within the first 5 min to 4 h post noise exposure, the earliest changes in the maximal damage area included deformation of the stereociliary bundle and swelling of the afferent dendrites below the IHCs. During the ensuing hours, swelling and distortion occurred in the OHCs, IHCs, pillar cells, and phalangeal cells. Complete degeneration of OHCs, IHCs, and pillar cells were observed at day-5 after exposure

and continued over time. The recovery of the afferent dendritic swelling was observed by 24 h after noise exposure. This was confirmed by later studies such as Peul et al. 1998. But surprisingly, the spiral ganglion neuron degeneration was still seen under light microscopy at the week-4 post exposure. In fact, the swelling and distortion of the organ of Corti, as was seen earlier, was also seen at week-4 point, suggesting active processes of both degeneration and repair. In the findings of Hamernik and Henderson, a considerable time delay on the order of 5 days to several weeks was observed before hair cell loss peaked and then stabilized following exposure to impulse noise (Hamernik and Henderson, 1974; Henderson and Hamernik, 1986). The mechanism by which this ongoing degeneration occurs weeks after the initial insult is not fully elucidated, but has important implications in terms of potential rescue therapy.

Noise is a pervasive and increasing hazard in the environment. Davis et al. (1935) found that a minimum sound pressure level of 95 decibels (dB) was necessary to induce auditory damage. Decibels describe the logarithmic ratio of the intensity of a given sound to that of a sound which is just perceptible to a person with normal hearing. Thus, a doubling of sound intensity will result in an increase of 3 dB. Humans can hear sounds with frequencies over the range 20 Hz to 20 kHz. Because of the shape of the external ear canal and other factors, the human's sensitivity to sound is greatest between 1 and 5 kHz (May, 2000). Damage within the cochlea tends to occur initially and to the greatest degree in the portion that detects sound in the 3-4 kHz range. For workers exposed to potentially harmful noise levels, this

progresses steadily over the initial decade of exposure and then tends to plateau. Typically, the next affected area is in the 6 kHz region followed by the 8 kHz and the 2 kHz regions where losses are more slowly progressive (Taylor et al., 1965). Most workers will have a relatively symmetrical, bilateral sensorineural hearing deficit. In theory, this damage reflects both the intensity of the noise and the length of exposure in a fashion that is predictable. In reality, the degree of hearing loss is usually not linear with respect to exposure. However, after years of exposure to harmful noise, a great number of workers will reach the American Occupational Safety and Health Administration's definition of material impairment of hearing, which is an average threshold shift of $\geq$ 25 dB at 1, 2, and 3 kHz (May, 2000). Many affected people actually experience losses considerably beyond 25 dB and may have problems ranging from tinnitus to difficulty in detecting and recognizing sounds, in comprehending speech and localizing sound sources.

The auditory functional impairment can be divided into four classifications: (1) temporary threshold shift (TTS) (also referred as auditory fatigue) may occur after only a few minutes of exposure to intense noise and is reversible after a period of time away from the noise; (2) asymptotic threshold shift is the threshold shift that reaches asymptotic level after continuous noise exposure (hours to days) and can return to pre-exposure level after the end of the exposure; (3) compound threshold shift is one kind of threshold shift with both temporary and permanent components and does not return to normal level; (4) permanent threshold shift (PTS) is a stable threshold shift after the temporary component disappears.

Around 10% of the population suffers from hearing disorders. Noise trauma is one of the most common reasons of hearing disorders. It is important to understand the mechanisms that are involved in the hearing impairments for early detection and intervention of hearing loss. The susceptibility to noise trauma is related to several factors, such as species differences, age, pigmentation, anesthesia, and body temperature. Two main mechanisms have been proposed for noise-induced hearing loss (NIHL), the rapid onset of mechanical damage and the gradual onset metabolic disturbance (Saunders et al., 1985; Borg et al., 1995). The mechanical impairment occurs mostly during intense noise exposure, which depends on the frequency, intensity and the duration of exposure, while the metabolic damage may be the result of enzyme alteration and ion concentration changes inside the cells after noise stimulation.

Excitotoxicity and oxidative stress in NIHL

The term excitotoxicity was first described by Olney (1978), referring to a process of neuronal death caused by excessive or prolonged activation of receptors for the excitatory amino acid neurotransmitter glutamate. Excitotoxicity plays an important role in many central nervous system (CNS) diseases, such as CNS ischemia, and CNS trauma (Doble, 1999). Under these pathological conditions, glutamate is excessively released to the synapse and binds to its receptors on neuronal cells (Fig. 9). The process of excitotoxicity is characterized by two main elements: depolarization of neurons with Na^+ influx and the entry of extracellular Ca^{2+} into

neuronal cells. Depolarization is primarily initiated by activation of AMPA receptors and subsequently the voltage-dependent Na^+ channels. The entry of Na^+ is followed by a passive entry of Cl^- and water, resulting in an increase in cellular volume and acute neuronal swelling. This osmotic component is potentially reversible if the stimulus is removed (Choi, 1987). If the stimulus remains, the continuous depolarization will release the magnesium blockage of the NMDA receptor, leading to the opening of the NMDA receptor. The elevated extracellular glutamate causes the influx of Ca^{2+} into neuronal cells through the opened NMDA receptors. Intracellular Ca^{2+} will also rise due to impaired activity of the membrane Na^+/Ca^{2+} exchanger (Koch and Barish, 1994). The increased intracellular free Ca^{2+} will stimulate the activity of numerous enzymes and trigger other calcium-dependent protein-protein interactions that are ultimately deleterious to cell homeostasis, and thus will lead to neuronal death (Doble, 1999).

The oxidative stress is referred to the imbalance between cellular production of free radicals and the ability of cells to efficiently defend against them (Simonian and Coyle, 1996). A free radical is any chemical species that contains one or more unpaired electrons, which make it more reactive because they tend to cause other molecules to donate their electrons (Halliwell and Gutteridge, 1989). The most common cellular free radicals are hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{-\bullet}$), and nitric oxide ($NO^{\bullet}$) (Simonian and Coyle, 1996). Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrate (ONOO), are not free radicals, but can

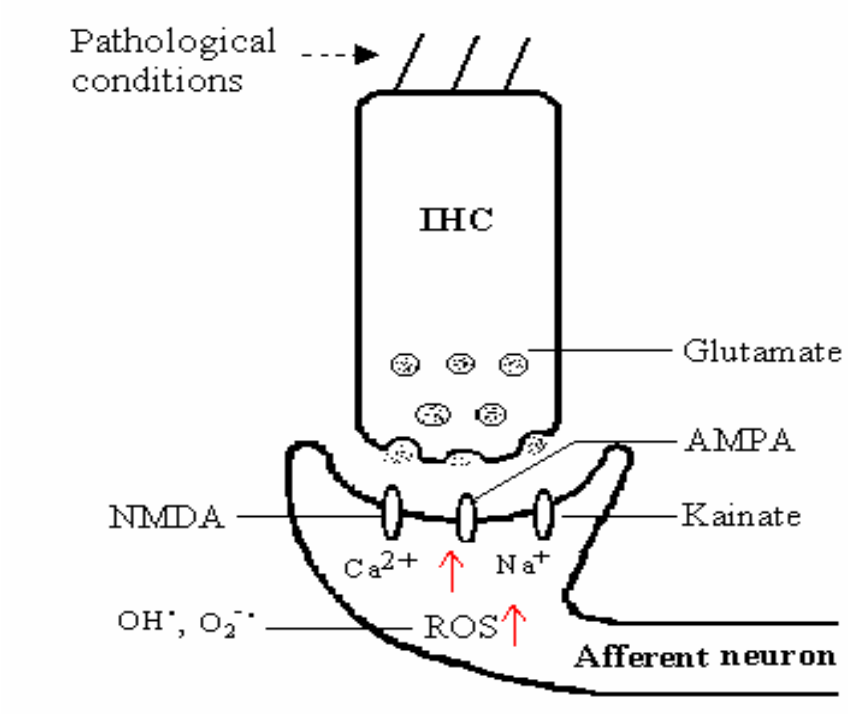


Fig. 9. The excitotoxicity and/or oxidative stress are involved in the pathophysiology of certain inner ear disorders, such noise-induced hearing loss, sudden hearing loss, and neural presbycusis. Pathological stimulations will cause the over-release of glutamate and/or over-production of ROS. Glutamate may increase ROS production, and on the other hand, ROS may induce glutamate release, suggesting they may have bi-direction relationship.

lead to their generation through various chemical reactions. Free radicals and related molecules are often classified together as reactive oxygen species (ROS) to signify their ability to promote oxidative changes within the cell (Simonian and Coyle, 1996). Cells normally employ a number of defense mechanisms against damage induced by free radicals (Evans, 1993; Simonian and Coyle, 1996). Problems occur when production of ROS exceeds their elimination by the natural antioxidant defence system, or when the later is damaged (Fig. 9). The increasing ROS production will deplete cellular antioxidant defenses and cause various

radical-mediated damage to lipid, proteins and DNA, leading to cellular damage and subsequently cell death (Doble, 1999).

The relationship between excitotoxicity and oxidative stress has not been well established. In the central nervous system, it has been proposed that excitatory amino acid (mainly glutamate) and ROS may cooperate in the pathogenesis of neuronal damage (Bose et al., 1992). Excitatory events can stimulate ROS, and ROS may lead to glutamate release, suggesting a bi-direction relationship (Pellegrini-Giampeitro et al., 1990). Following transient ischemia, the cerebral levels of excitatory amino acid and free radicals were both increased (Delbarre et al., 1991). During excitotoxicity, the increased intracellular calcium can activate calcium-dependent enzymes, such as phospholipase A_2, nitric oxide synthase, and xanthine oxidase, leading to the generation of ROS (Doble, 1999). Exposure of mitochondria to high concentration of ambient calcium results in a surge of free radical production (Dykens, 1994). On the other hand, the ROS scavengers, such as D-mannitol and indomethacin, can reduce ischemia induced excitatory amino acid production. Furthermore, the incubation of hippocampal slices with systems leading to free radical formation resulted in an increase of the release of endogenous glutamate and aspartate (Pellegrini-Giampeitro et al., 1990).

In the auditory system, significant glutamate efflux from the IHCs has been demonstrated under stimulus conditions in both *in vitro* and *in vivo* studies [Bledsoe et al., 1980; Jäger et al., 1998, 2000]. Bledsoe et al. (1980) showed greater

glutamate efflux from stitch skins compared to non-stitch skins containing the lateral-line organ in Xenopus laevis. In an *in vitro* isolated temporal bone preparation, Jäger et al. (1998) demonstrated noise stimulus induced the increases in the levels of glutamate and aspartate. Furthermore, they found significant increase of glutamate and aspartate in the scala tympani of guinea pig cochlea by using *in vivo* microdialysis before and during noise exposure. As in other parts of the nervous system, the excessive glutamate in the cochlea after noise stimulation will have excitotoxicity to the afferent neurons, leading to the acute neuronal swelling and later on neuronal cell death. Indeed, application of glutamate agonists has been shown to induce destruction of primary auditory dendrites and to alter cochlear function in a fashion similar to that observed after acoustic trauma [Spoendlin, 1971; Robertson, 1983; Pujol et al., 1985; Puel et al., 1994; Duan and Canlon, 1996].

There is accumulating evidence that increased ROS production and their ototoxicity are involved in the NIHL (Kopke et al., 1999). Direct evidences are derived from the findings that: (1) $O_2^{-\bullet}$ radicals emerge in the stria vascularis after noise exposure (Yamane et al., 1995); (2) $OH^{\bullet}$ significantly increases in the cochlea early following intense sound stimulus (Ohlemiller et al., 1999); (3) ROS affected the morphology of isolated OHCs or damaged cochlear function following perilymphatic space infusion (Cleric et al., 1995; Cleric and Yang, 1996). Indirect evidences are found by the findings: (1) the activity of some antioxidant enzymes increases during conditioning noise exposure which reduces NIHL (Jacono et al., 1998); (2) the

endogenous antioxidant, glutathione, is upregulated in the lateral wall following noise exposure (Yamasoba et al., 1998a), and in contrast, the reduction of glutathione increases NIHL (Yamasoba et al., 1998b); (3) a variety of antioxidants, such as superoxide dismutase-polythylene glycol and allopurinol, can attenuate NIHL (Seidman et al., 1993).

Protection of auditory function with glutamate receptor antagonist and antioxidant

Glutamate mediated toxicity plays a crucial role in NIHL. There are several sites to attenuate the glutamate-induced excitotoxicity. One site is to reduce the glutamate synthesis and release from the pre-synapse or to increase its uptake by glutamate transporters. Another site is to antagonize the excessive glutamate at receptor level with glutamate receptor antagonists. Finally, drugs could be used to offset the intracellular and extracellular neurotoxic events set in motion by receptor overstimulation. All these approaches have met with certain success in vitro, but many of the drugs used have unacceptable clinical side-effects (Choi, 1988). Glutamate receptor antagonists have been widely investigated for the neuroprotection against excitotoxicity in both *in vitro* and *in vivo* studies. Pingle et al. (1997) showed that CNQX, a non-NMDA receptor antagonist, and MK-801, an NMDA receptor antagonist, when applied pre-insult or immediately post-insult, were able to prevent neuronal death of CA1 pyramidal cells *in vitro* caused by either hypoxia or ischemia. The neuroprotective effect of MK-801 was demonstrated *in vivo* in the rat middle cerebral artery occlusion model of focal

ischemia (Gill et al., 1991). Solberg et al. (1997) demonstrated that MK-801 significantly reduced photoreceptor-cell loss in retinal laser injury.

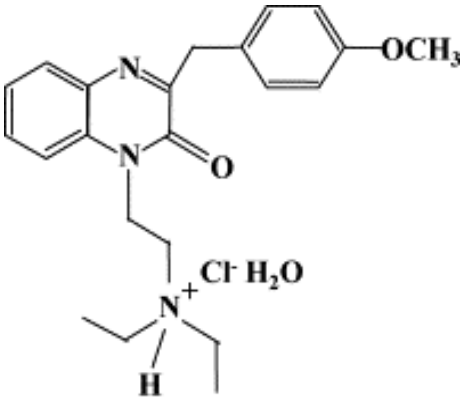
The protective role of glutamate receptor antagonist has also been investigated in the auditory system. Janssen (1992) showed that the broad-spectrum antagonist kynurenic acid or MK-801 could prevent high-frequency hearing loss caused by glutamate. In another study, the AMPA/kainate receptor antagonist DNQX was found to prevent most of both AMPA-induced and ischemia-induced dendritic swelling, while the combination of DNQX and D-AP5, an NMDA receptor antagonist, resulted in a nearly complete protection of all the dendrite (Puel et al., 1994). MK-801 or kynuretic acid has been demonstrated to prevent noise-induced dendritic damage beneath the IHCs (Puel et al., 1998; Duan et al., 2000). Chen et al. (2001) found MK-801 significantly reduced the permanent compound action potential threshold shifts induced by noise trauma in rat.

Since ROS are important causative factors in the NIHL, a variety of antioxidants have been shown to effectively attenuate hearing loss after noise exposure. Seidman et al. (1993) found that superoxide dismutase-polyethylene glycol, a scavenger of ROS, and allopurinol, a blocker of ROS production and potential scavenger of ROS, could attenuate noise-induced threshold shift. The animals treated with lazaroids, lipid peroxidation inhibitors and ROS scavengers, showed less noise-induced cochlear action potential threshold shifts and cochlear microphonic when compared to non-drug treated noise-exposed subjects (Quirk et al., 1994). The

cochlear function was significantly protected from noise with the antioxidant mannitol and the iron chelator deferoxamine mesylate (Yamasoba et al., 1999). Ohinata et al. (2000) showed that the cellular antioxidant glutathione could significantly limit the noise-induced cochlear damage.

Caroverine is a glutamate receptor antagonist and antioxidant

Caroverine (Spasmium®, Phafag AG), 1-(2-diethylaminoethyl)-3-(*p*-methoxy benzyl)-1,2-dihydro-2-quinoxalin-2-on-hydrochloride (Fig. 10), is chemically derived from isoquinoline, the basic structure of papaverin. It is clinically available in some countries as a spasmolytic drug based on its unspecific Ca^{2+}-channel blocking activity for more than 40 years (Hornykiewicz et al., 1963). Microiontophoretic experiments in guinea pigs have demonstrated that caroverine exhibits competitive AMPA antagonism, and at higher concentrations, non-competitive NMDA antagonism in the cochlear afferents (Ehrenberger and Felix, 1992). Recently, Udilova and his colleagues (2003) found strong antioxidant



$C_{22}H_{28}N_3O_2Cl \cdot H_2O$

Fig. 10. Chemical structure of caroverine-hydrochloride. It is a quinoxaline derivative.

activity of caroverine based on both its partial prevention and highly active scavenging on $OH^{\bullet}$ radical, which is by far the most potent and dangerous oxygen metabolite (Udilova et al., 2003). As a glutamate receptor antagonist together with antioxidant activity, caroverine has a variety of beneficial results in the treatment of tinnitus, sudden hearing loss and other neurotoxic effects in the inner ear (Ehrenberger, 2002). In a study on sixty patients suffering from tinnitus with a probable cochlear origin, a dose of 160 mg caroverine in physiological saline solution was infused to patients at the rate of 2-3 ml/min (Denk et al., 1997). Both a subjective rating of the tinnitus on a five-point scale (0 = no tinnitus; 1 = slight; 2 = moderate; 3 = severe; 4 = tormenting tinnitus) and a psychoacoustic measurement of tinnitus as tinnitus matching were carried out for the evaluation of tinnitus before, immediately after and 1 week after treatment. 63.3% of the patients responded positively to the single infusion of caroverine. The beneficial effect of the caroverine therapy was still present after 1 week in 43.3% of the patients (Denk et al., 1997).

Since both excitotoxicity and oxidative stress play important roles in NIHL, and their relation is not well elucidated, a drug, such as caroverine, with both antiglutamatergic and antioxidant activities seems promising for the protection and treatment against NIHL. Although the safety of caroverine has been documented (Koppi et al., 1987; Saletu et al., 1995), the potential adverse effects of glutamate receptor antagonist should be considered when applied systemically (Muir and

Lees, 1995). In addition, the therapeutic effect of systemic caroverine may not be ideal at non-toxic doses because of its limited ability to penetrate the blood-labyrinth barrier. Therefore, local application of caroverine onto the gelfoam placed on the RWM might be an alternative to achieve therapeutic concentration in the inner ear and avoid systemic side effects. For clinical application, the choice of caroverine should be based on its pharmacokinetic behavior in the inner ear fluids. Basic information about the rate of drug diffusion across the RWM, systemic caroverine absorption, and elimination of drug from the inner ear is necessary. The drug concentration in the perilymph after systemic administration at the dose used in clinic would be of interest because we might assume it to be the therapeutic drug level in the inner ear.

Aims of the study

1. Study the pharmacokinetics of caroverine in the preilymph, CSF and plasma following systemic and local RWM applications in the guinea pig at regular time intervals. The RWM application might bypass the blood-labyrinth barrier to achieve higher inner ear drug concentration and avoid systemic disturbance. The concentrations of caroverine in the perilymph, CSF and plasma will be determined by high performance liquid chromatography method. The RWM administrations with two different doses, one low dose and another high dose, will be compared with the systemic application. The peak caroverine concentration and the elimination of the drugs in the three compartments will be monitored. The differences in the concentration of these three compartments will be compared. The caroverine concentration in the perilymph following systemic application at the dose used in clinic might be assumed as effective therapeutic caroverine concentration in the perilymph.

2. Study the effect of caroverine on auditory function following local RWM applications in the guinea pig. As an NMDA and AMPA receptor antagonist, caroverine might block the neurotransmitter glutamate's physiological function and decrease the hearing sensitivity. The auditory brainstem response will be measured following the RWM applications at regular time intervals. The extent of the auditory functional effect will be known from the ABR threshold shift. The

important issue is that whether the auditory effect is reversible and in what time course. This will also dermine the limination of RWM application.

3. Study the protective role of caroverine in the NIHL following local RWM applications in the guinea pig model. With the antiglutamatic and antioxidant activities, caroverine might be able to to protect the cochlea against excitotoxicity and oxdative stress produced by noise trauma. The RWM applications with one low dose of caroverine and another high dose and saline as control will be performed before noise exposure. The ABR will be measured following noise exposure at regular time intervals to monitor the recovery of the hearing. The protective effect of caroverine against noise trauma will be determined by the comparison of hearing recovery between caroverine groups and control group. The low and high dose group will also be compared to show whether the protective effect is dose-dependent.

4. Study the therapeutic role and time window of caroverine in the NIHL following local RWM application in the guinea pig model. The time course of metabolic damages, such as excitotoxicity and oxidative stress, in the noise-induced hearing loss following noise exposure is not well known. Caroverine might interfere with this kind of impairmet whenever it continues. The caroverine will be applied to the RWM at 1 h or 24 h after noise exposure. The ABR will be measured at regular time interval following the recovery period of the cochlea.

MATERIALS AND METHODS

Pharmacokinetics study

Animals

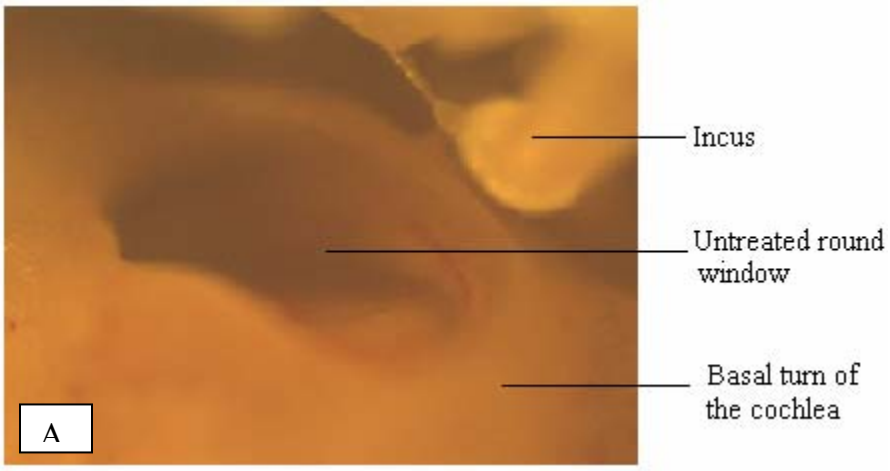
Albino guinea pigs of either sex (300 -- 400 g) were used in this study. The care for and use of the animals were approved by the Ethical Committees at the National University of Singapore and the Karolinska Institutet in Stockholm. Totally 45 animals were randomly assigned to 3 groups: 1 group for intravenous injection (IV) and 2 groups for local applications onto the RWM with a low dose (LD) and the other high dose (HD) as shown in the table 1. The animals were anesthetized by intramuscular injection with a mixture of ketamine (40 mg/kg) and xylazine (4 mg/kg).

Table 1. Totally 45 guinea pigs were devided into three groups: IV, LD and HD groups with 15 animals in each group. The plasma, CSF and perilymph samples were collected at 5 time courses with 3 animals at each time course.

IV group		**LD group**		**HD group**	
Time course	**Animal number**	**Time course**	**Animal number**	**Time course**	**Animal number**
10 min	3	10 min	3	10 min	3
30 min	3	30 min	3	30 min	3
60 min	3	60 min	3	60 min	3
180 min	3	180 min	3	180 min	3
360 min	3	360 min	3	360 min	3

Systemic and local caroverine applications

Intravenous injection was administered via the right femoral vein with the dose of 4 mg/kg body weight at the concentration of 1.6 mg/ml in physiological saline. For local applications, two doses were used: 15 µl of 1.6 mg/ml (LD) and 12.8 mg/ml (HD) of caroverine in physiological saline. Under an operating microscope, the right temporal bulla was opened through a post-auricular incision to expose the round window under aseptic conditions. The RWM was examined under microscopy to make sure that the RWM was clean and intact before drug administration (Fig. 11A). The round window membrane was seldom damaged by the surgery. We discarded the animals when we found the round window membrane was not intact. A small piece of gelfoam was placed on the RWM. Fifteen microliters of caroverine at the concentrations of either 1.6 or 12.8 mg/ml were dropped on the gelfoam (Fig. 11B). The hole of the temporal bulla was then closed using dental cement (Fuji I, Japan) and the skin sutured.



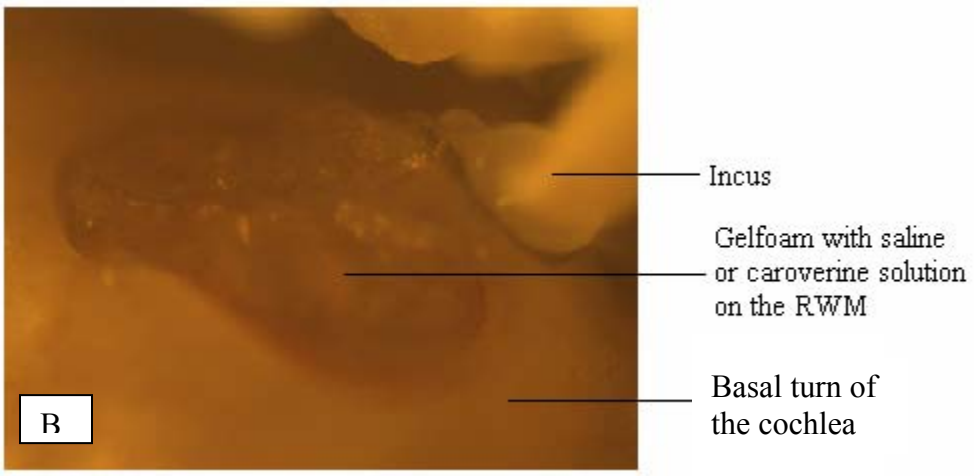


Fig. 11. A. The round window after cleaning the fluid and mucosa from the round window membrane. B. One piece of gelfoam was place onto the round window membrane and 15 µl of physiological saline or caroverine solution was dropped onto the gelfoam.

CSF, plasma and perilymph sampling

CSF, plasma and perilymph were sampled at 10, 30, 60, 180 and 360 min after systemic or local caroverine administrations. Three animals were included at each time point (15 animals in each group, total of 45 animals).

CSF sampling. The animals were anesthetized with the mixture of ketamine and xylazine as above, and in addition, local anesthesia with xylocaine was given. An incision was made through the skin and muscle of the dorsal neck to expose the arachnoid. Twenty microliters of CSF were collected from the subarachnoid space at the foramen magnum with a 1-ml syringe connected to a 29-gauge needle.

Plasma sampling. After CSF sampling, a 3-ml blood sample was collected by heart puncture through the thoracic cavity using a 5-ml syringe. The plasma was obtained by centrifugation of the blood sample at 3,000 rpm for 5 min.

Perilymph sampling. In order to avoid CSF contamination, the animals were deeply anesthetized with the mixture of ketamine and xylazine, the perilymph was then collected after decapitation (Fig. 12). The bulla was removed from the skull base and opened to expose the middle ear. After removing the remaining gelfoam, the RWM and middle ear cavity were rinsed 4 times with 30% methanol within 2 min under an operating microscopy. To make sure that there was no contamination of the perilymph by the residual caroverine in the middle ear cavity, the last wash was collected for detection of caroverine using high-performance liquid chromatography (HPLC), and only perilymph samples with no detectable caroverine in the last wash were used for analysis. Six to 10 μl of perilymph were collected through the RWM with a glass-capillary after complete removal of the solution in the middle ear cavity. In the ears of the animals given the drug systemically, the samples were obtained in the same way without local RWM rinsing. All of the samples were stored at –20°C for HPLC analysis within 1 week.

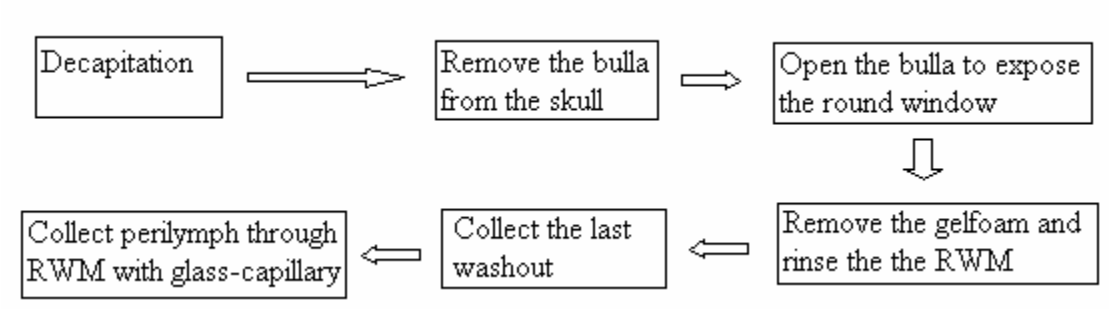


Fig. 12. The procedure of the perilymph sample collection.

HPLC analysis

The concentration analysis was performed on a Hewlett Packard (HP) 1050 HPLC system equipped with chemstation and HP 1100 UV detector set at 230 nm. A guard column (4.6 × 12.5 mm, 5 μm, HP) was connected to the HPLC column (Hypersil BDS C18, 5 μm, 150 × 4.6 mm). Drug concentrations with samples were determined from calibration curves obtained by plotting the chromatographic peak area ratio of caroverine/internal standardization described below. Peak areas were computed from the HP HPLC system software chemstation.

The mobile phase for caroverine in the perilymph and CSF was 34% of acetonitrile and 66% of the mixture of 0.02 mol KH_2PHO_4 and 1.5 ml of diethylamine in 1 liter of deionized water adjusted to pH 5.7 with 1 *N* HCl. For caroverine in plasma, the mobile phase was 30% of acetonitrile and 70% of the mixture of 0.02 mol KH_2PHO_4 and 1.5 ml of diethylamine in 1 liter of deionized water adjusted to pH 5.9 with 1 *N* HCl. The flow rate was 1 ml/min. Standard stock solutions of caroverine-hydrochloride and flunitrazepam (internal standard; both 1 mg/ml) were prepared in 100% methanol and stored at 4°C for not more than 2 weeks. Calibration samples were prepared at different concentrations by diluting the stock solution with normal saline. At least 5 caroverine calibrators in 20 μl saline, 20 μl of control CSF samples or 10 μl of control perilymph samples (diluted to 20 μl with normal saline) were mixed with 10 μl of flunitrazepam of appropriate concentrations. The mixture was vortexed and directly injected into the HPLC system. Different calibration curves had to be used because of the wide range of

caroverine concentrations in the perilymph (calibration range: from 10 ng/ml to 9.6 μg/ml). Linear calibration curves were obtained. For caroverine in plasma, calibration samples were prepared in 100 μl of control plasma samples. Two hundred microliters of acetonitrile and 20 μl of flunitrazepam of appropriate concentrations were added to 20 μl of caroverine hydrochloride calibrators (calibration range: 10--960 ng/ml) and 100 μl of control plasma samples. The mixture was centrifuged to precipitate plasma proteins. After centrifugation, the supernatant was evaporated with a stream of nitrogen air and the residue was reconstituted in 40 μl of mobile phase for injection. The retention times of caroverine and flunitrazepam were 13.1 and 18.0 min, respectively, in plasma, and 8.6 and 11.9 min, respectively, in the perilymph and CSF. The interday coefficient of variation ranged from 4.6 to 16.1%. Some figures of the wave forms were illustrated in Fig. 13.

Using the above conditions, the limit of quantification was 10 ng/ml. This HPLC method for analysis of caroverine in the perilymph, CSF and plasma is reproducible and sensitive. No interference from endogenous substances or the anesthetic agents was encountered.

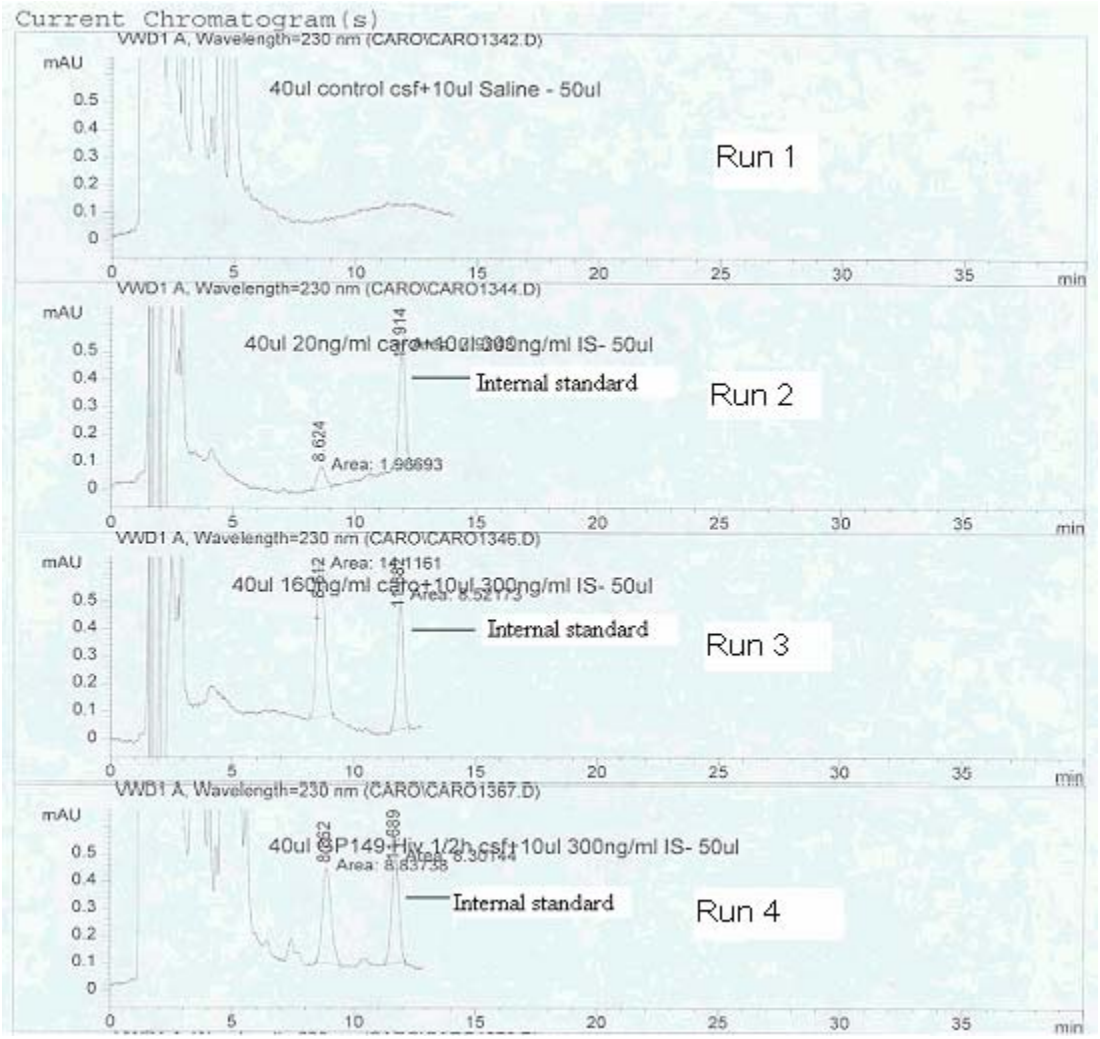


Fig. 13. Some examples of the HPLC figures. In the Run 1, 40 µl of control CSF from untreated guinea pig and 10 µl of physiological saline did not produce any peak between minute-6 and minute-14. In Run 2, 3 and 4, 10µl of internal standard solution at 300 ng/ml were added and produced a peak at minute-11.9 with similar area. In Run 2 and 3, 40 µl of caroverine in control CSF from untreated guinea pig at the concentration of 20 ng/ml or 160 ng/ml were added and peaks with different areas appeared at minute-8.6. In the Run 4, the CSF sample from the guinea pig with intravenous caroverine treatment produced a peak at minute-8.6 and the area of the peak could be measured.

Auditory functional effect following local RWM applications

Animals and local RWM applications

For the animals with local caroverine applications, ABR measurements were performed 1 day before, and 30 min, 3, 6 and 24 h after application. Fifteen animals were randomly assigned to 3 groups: LD and HD local groups and a control group. Each group included 5 animals. The animals were anesthetized as above. In the control group, 15 μl of normal saline was applied onto the RWM with the gelfoam. In the LD and HD local groups, the caroverine administrations were the same as in the pharmacokinetic study.

ABR measurements

ABR measurements were performed in a soundproof booth as described previously (Duan et al., 2000). Responses were recorded with subcutaneous stainless electrodes as the potential difference between an electrode on the vertex and an electrode behind the ear, the same side hind leg serving as the earth (Fig. 14). The body temperature of the animals was maintained at 38°C by using an isothermal heating pad. Stimulus intensity was calibrated with a ¼-inch condenser microphone (Bruël & Kjær Instruments, Marlborough, Mass., USA, model 4135) and all of the sound pressure levels (SPL) were expressed in decibels relative to 20 μPa. The stimulus signal was generated through Tucker-Davis Technologies (Gainesville, Fla., USA) equipment controlled by a computer and delivered by an earphone (Telephonics TDH 39, Farmingdale, N.Y., USA). The stimuli were delivered

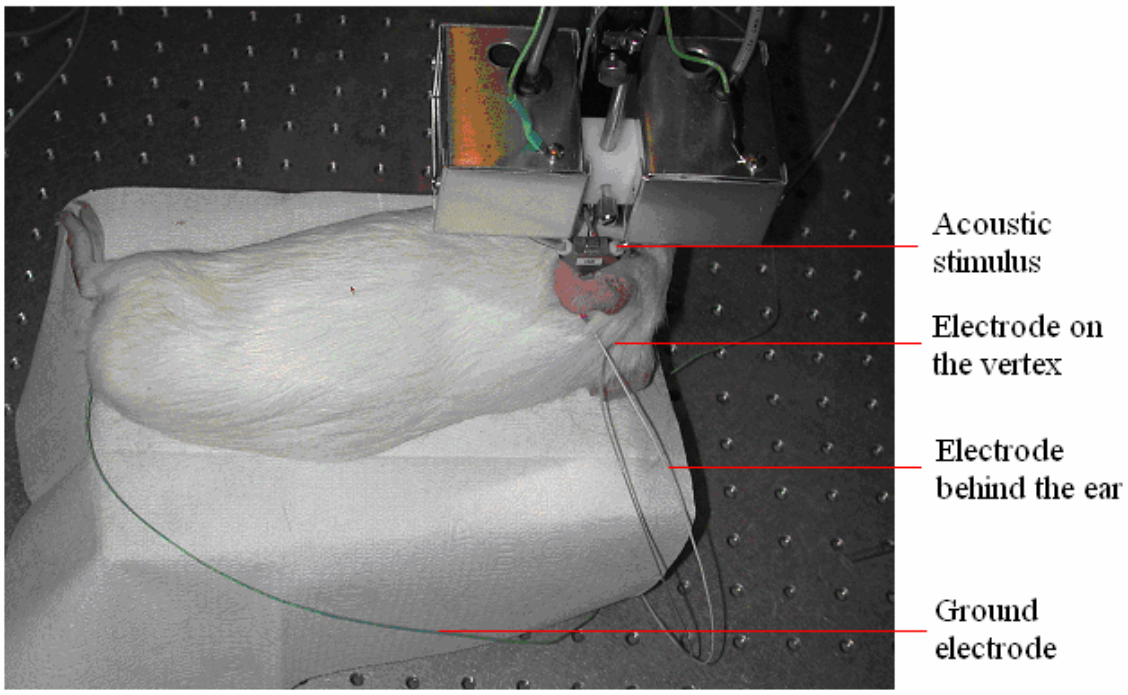


Fig . 14. ABR responses were recorded with subcutaneous stainless electrodes as the potential difference between an electrode on the vertex and an electrode behind the ear, the same side hind leg serving as the earth. The stimuli were delivered through a closed acoustic system with one end connected to the earphone and the other end sealed into the external auditory meatus.

through a closed acoustic system with one end connected to the earphone and the other end sealed into the external auditory meatus. The evoked response was amplified 100,000 times and averaged 2048 sweeps in real time by a digital signal processor (DSP32C) with a time domain artifact rejection. The initial intensity of the stimulus was 110 dB peak SPL and was then decreased in 10-dB steps until the threshold was approached and then in 5-dB steps until the ABR disappeared. The threshold was defined as the lowest intensity at which a visible ABR wave III was seen in two averaged runs since the wave III was the largest wave in guinea pigs. Threshold was measured at 4 frequencies: 20, 16, 12.5 and 8 kHz. The latency and amplitude of wave I at 90 dB of each frequency were recorded. One-way repeated

measures analysis of variance (ANOVA) was used to determine if there was a significant effect of caroverine treatment on the mean values of thresholds, latencies and amplitudes, followed by Tukey test for significance versus the control group at specific frequencies.

Protection of auditory function against noise trauma with local caroverine administration

Animals and local RWM administrations

Eighteen pigmented guinea pigs of either sex (300 -- 400 g) were randomly divided into 3 groups: low dose (LD) and high dose (HD) caroverine groups and a control group, with 6 animals in each group. The animals were anesthetized with the mixture of ketamin and xylazine as above and the surgical procedures of RWM applications were the same as above. The control group received 15 μl of physiological saline with gelfoam, the LD group 15 μl of 1.6 mg/ml of caroverine in physiological saline with gelfoam, and the HD group 15 μl of 12.8 mg/ml of caroverine in physiological saline with gelfoam. The hole of the temporal bulla was then closed using dental cement (Fuji I, Japan) and the skin sutured.

Noise exposure

Ten min after the RWM administrations of physiological saline or caroverine, the anesthetized animals were transferred to a sound proof booth to expose to one-third-

octave band noise centered at 6.3 kHz (110 dB SPL) for 1 hour. The sound proof booth was equipped with a speaker horn (model 2328, James B. Lancing Sound Inc. Los Angeles. CA, USA) mounted in the ceiling. The free field noise exposure was generated with software from Brüel & Kjær (Pulse) and delivered by a sound generator (Brüel & Kjær LAN Interface Module type 7533, Input/Output Module type 3109) connected to an amplifier (Brüel & Kjær type 2716). A small paper box with the same height as the guinea pig's ear was placed in the cage. The noise intensity (110 dB SPL) was measured prior to exposure using a ½ inch microphone (Brüel & Kjær type 4190) and a preamplifier (Brüel & Kjær type 2669C) on the paper box at the level of the animal's experimental ear. The microphone was placed on the position where the experimental ear would be and then started to generate the sound. The sound level could be read from the computer and the sound level could be adjusted from the program to reach 110 dB. Then the box was removed and replaced by the guinea pig with its experimental ear at the same level of the box. The contralateral ear was also exposed to the noise. But the sound intensity was not exactly the same as that of the experimental ear because the sound level varied with different positions.

ABR measurements and cochlea examinations

The ABR thresholds were obtained 1 day before, and 1.5 h (20 min after noise exposure), 3, 6, 24 h, 3 days and 1 week after RWM applications. ABR measurement was performed under the same condition and procedure as above. Thresholds were measured at 5 frequencies: 20, 16, 12.5, 8 and 4 kHz. After the

terminal ABR measurement, the animal was decapitated after giving an overdose of pentobarbital and the bulla was removed from the skull and opened to examine the middle ear and the round window under microscopy. Then the cochlea was put into 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and a small hole was made into the cochlear apex in order to examine if there was any damage, which could not be observed under the operating microscope. A plastic pipette was used to perfuse the cochlea with 4% paraformaldehyde gently from the opening in the cochlear apex so that any small hole on the round window membrane could be found under microscope. No obvious sign of inflammation was found in the middle ear or round window. One-way repeated measures analysis of variance (ANOVA) was used to test if there was a significant effect of caroverine treatment on the mean values of thresholds, followed by Tukey test for significance versus the control group at specific frequencies.

Therapeutic effect and time window on noise trauma with local RWM caroverine application

Animals and noise exposure

Pigmented guinea pigs of either sex (300 -- 400 g) were used. The animals were anesthetized with the mixture of ketamine and xylazine as above. The anesthetized animals were exposed to one-third octave band noise centered at 6.3 kHz (110 dB SPL) for 1 h in a sound proof booth, as described above.

Local caroverine or physiological saline applications

Twenty-four animals were randomly divided into 4 groups (6 animals in each group): 1 control and 1 caroverine group in which local RWM applications were performed 1 h after the end of noise exposure, and another 1 control and 1 caroverine group in which local administrations happened 24 h after noise exposure. Fifteen microlitres of either physiological saline as control or 12.8 mg/ml of caroverine solution were applied locally onto the RWM with gelfoam. The surgical procedure of local RWM application was the same as in pharmacokinetics study.

ABR measurements and cochlea examinations

The ABR thresholds were obtained 1 day before noise exposure, and at 0.5, 24 h, 3 days and 1 week after normal saline or caroverine RWM applications. The ABR measurement of anesthetized animal was performed in a sound proof booth as described above. The thresholds were measured at five frequencies: 20, 16, 12.5, 8 and 4 kHz. After the terminal ABR measurement, the animal was decapitated after giving an overdose of pentobarbital and the bulla was removed from the skull and opened to examine the middle ear and the round window under microscopy as mentioned above for inflammation. No obvious sign of inflammation was found in the middle ear or round window. The differences in mean values of threshold shifts between caroverine group and control group were tested for significance ($p<0.05$) by Student's two-tailed t-test.

RESULTS

Pharmacokinetics of caroverine

The mean caroverine concentrations ± SD in μg/ml in the perilymph, CSF and plasma following systemic and local RWM applications are shown as a function of time in Tables 2-4 and Fig.15-17. Local administrations resulted in dramatically higher level of perilymph caroverine concentration than that seen after intravenous injection (Fig. 15). The perilymph peak values were obtained at 30 min after caroverine applications in all three groups and then decreased with time. Peak perilymph concentration was 4.3 μg/ml in the LD group, and 18.8 μg/ml in the HD group. They were 0.27% and 0.15% respectively of the administered concentrations, which were 1.6 mg/ml and 12.8 mg/ml. Caroverine became undetectable at 6 h in the LD group, while the concentration still remained at 1.9 μg/ml at 6 h in the HD group. In the IV group, perilymph caroverine reached its peak value of 0.24 μg/ml at 30 min and became undetectable at 3 h after administration.

Table 2. The concentrations (mean ± SD, μg/ml) of caroverine in the perilymph, CSF, and plasma following high dose (15 μl of 12.8 mg/ml) RWM administrations. In the perilymph, the caroverine reached it highest concentration at 18.78 μg/ml at 30 min following application and decreased with time. It still remained at 1.93μg/ml at 6 h after application. In the CSF, caroverine was undetectable at 10 min and 6 h and remained at very low level between 30 min and 3 h. In the plasma, caroverine concentration was around 0.03-0.06 μg/ml during the 6 h after RWM application.

Mins	Perilymph	CSF	Plasma
10	5.240 ±2.500	0	0.063 ±0.016
30	18.780 ±0.266	0.025 ±0.012	0.055 ±0.019
60	12.567 ±1.838	0.012 ±0.002	0.053 ±0.002
180	4.591 ±1.151	0.014 ±0.001	0.048 ±0.017
360	1.932 ±0.777	0	0.034 ±0.014

Table 3. The concentrations (mean ± SD, μg/ml) of caroverine in the perilymph, CSF, and plasma following low dose (15 μl of 1.6 mg/ml) RWM administrations. In the perilymph, the caroverine reached it highest concentration at 4.26 μg/ml at 30 min following application and decreased with time and became undetectable at 6 h after application. In the CSF, caroverine was undetectable at 10 min and 6 h and remained at very low level between 30 min and 3 h. In the plasma, caroverine concentration was around 0.02-0.06 μg/ml during the first 3 h after RWM application and was undetectable at 6 h.

Mins	Perilymph	CSF	Plasma
10	3.745 ±2.057	0	0.056 ±0.004
30	4.263 ±0.288	0.013 ±0.001	0.044 ±0.021
60	1.110 ±0.382	0.013 ±0.001	0.034 ±0.019
180	0.971 ±0.272	0.019 ±0.018	0.027 ±0.013
360	0	0	0

Table 4. The concentrations (mean ± SD, μg/ml) of caroverine in the perilymph, CSF, and plasma following intravenous injection (4 mg/kg body weight at the concentration of 1.6 mg/ml). In the perilymph, the caroverine reached it highest concentration at 0.23 μg/ml at 30 min following application became undetectable at 3 h. In the CSF, remained at very low level and became undetectable at 3 h. In the plasma, caroverine concentration reached peak value of 0.75 μg/ml at 10 min and decreased with time. At 6 h the caroverine concentration in the plasma was still 0.09 μg/ml.

Mins	Perilymph	CSF	Plasma
10	0.186 ±0.009	0.149 ±0.004	0.750 ±0.137
30	0.239 ±0.051	0.107 ±0.027	0.417 ±0.081
60	0.163 ±0.016	0.069 ±0.026	0.216 ±0.023
180	0	0	0.144 ±0.034
360	0	0	0.094 ±0.051

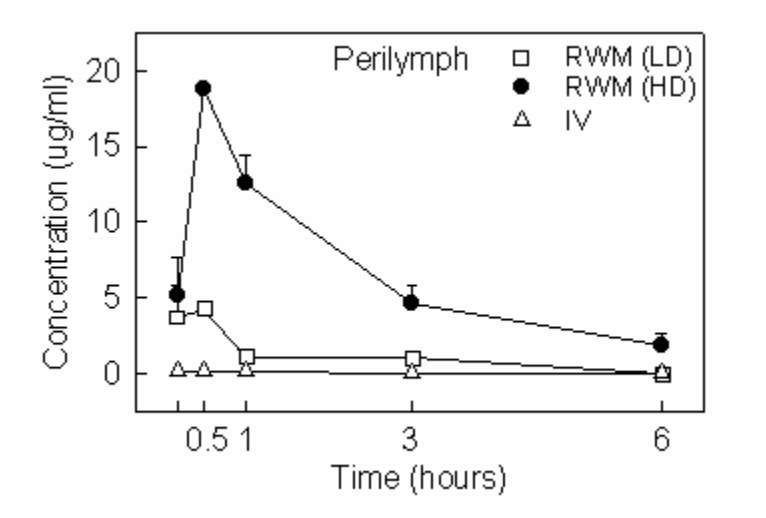


Fig. 15. The pharmacokinetic curves of caroverine in the perilymph after intravenous injection or RWM administrations. The caroverine concentration reached peak value at 30 min after application and decreased with time. The caroverine peak concentration in the HD group is dramatically higher than that in the IV group. At 6 h, caroverine became undetectable in the IV or LD group and still remained at high level in the HD group.

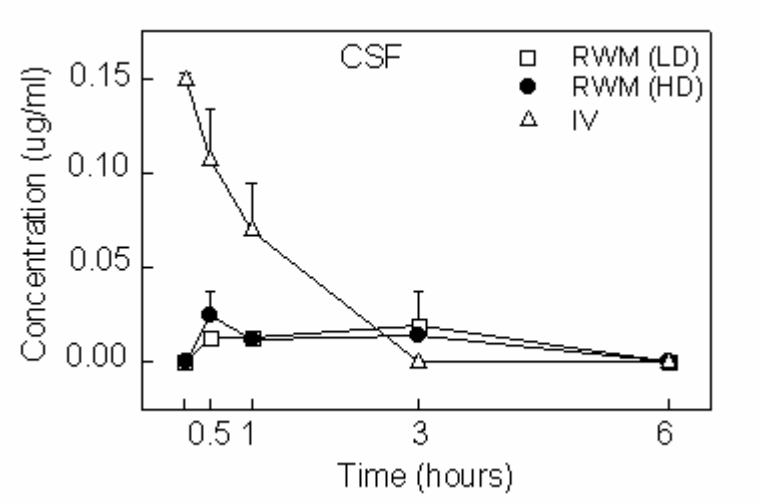


Fig. 16. The pharmacokinetic curves of caroverine in the CSF after intravenous injection or RWM administrations. The CSF caroverine concentration reached peak value at 10 min after systemic application and decreased with time. While in the local groups caroverine remained at much lower level compared with IV group.

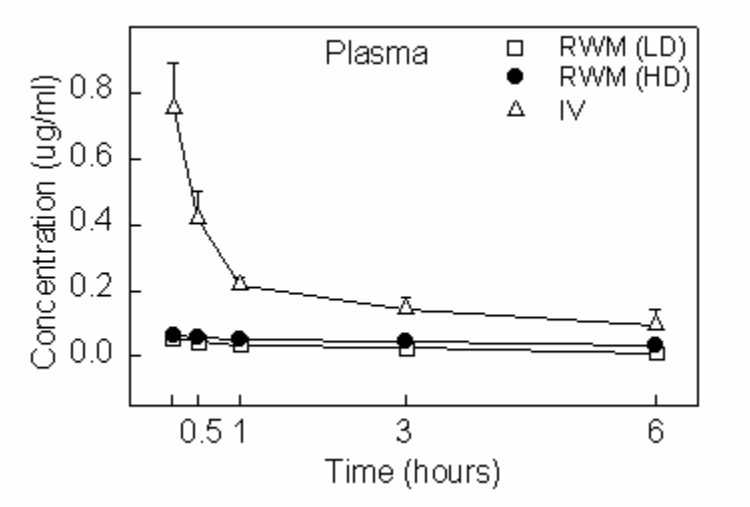


Fig. 17. The pharmacokinetic curves of caroverine in the plasma after intravenous injection or RWM administrations. The caroverine concentration reached peak value at 10 min after IV administration and decreased around 4 times during 1 h and remained at relatively stable high level. In both local groups, caroverine remained at low level in the plasma.

As shown in Fig. 16, caroverine in the CSF was detected in both local groups at 30 min after applications and remained at very low levels (ranging from 0.012-0.025 μg/ml) until it became undetectable at 6 h. In the IV group, CSF caroverine reached a much higher peak concentration (0.149 μg/ml) 10 min after administration and then decreased with time. There was no statistically significant difference ($p = 0.39$) in caroverine concentrations between the perilymph and CSF following IV administration. However, the concentration in the perilymph seemed to be relatively higher than in CSF.

The concentration of caroverine in plasma (Fig. 17) reached a peak value 10 min after both local and IV administrations of caroverine, and then decreased with time. However, caroverine was still detectable 6 h after IV administration. In both LD and HD groups, the concentrations were much lower than those in the IV group,

and became undetectable at 6 h in the LD group. The peak value of caroverine in the plasma in the IV group was around 11 times higher than that in the HD group.

The effect of local applications on auditory function

The effect of local applications on auditory function was evaluated by the measurements of ABR threshold, amplitude and latency. ABR thresholds at 4 different frequencies (20, 16, 12.5 and 8 kHz) are shown in Fig. 18. At 20 kHz, the threshold shifts at 30 min following RWM application were 8, 27 and 56 dB in the control, LD and HD groups, respectively. Comparing ABR threshold values post treatment with the pre-treatment values at 20 kHz, both LD and HD groups exhibited significantly impaired thresholds at 30 min, 3 and 6 h after application ($p = 0.009$, 0.001, 0.006; and $p = 0.001$, 0.001, 0.002, respectively). Comparison of ABR threshold shifts at 20 kHz in the LD and HD groups with the control group showed statistically significant differences at 30 min and 3 h ($p = 0.014$ and 0.002 for LD group; $p = 0.0003$ and 0.003 for HD group). The threshold shift was smaller at 16 and 12.5 kHz and the least at 8 kHz. All the thresholds recovered partially at 3 and 6 h and had completely returned to normal level 24 h after caroverine applications.

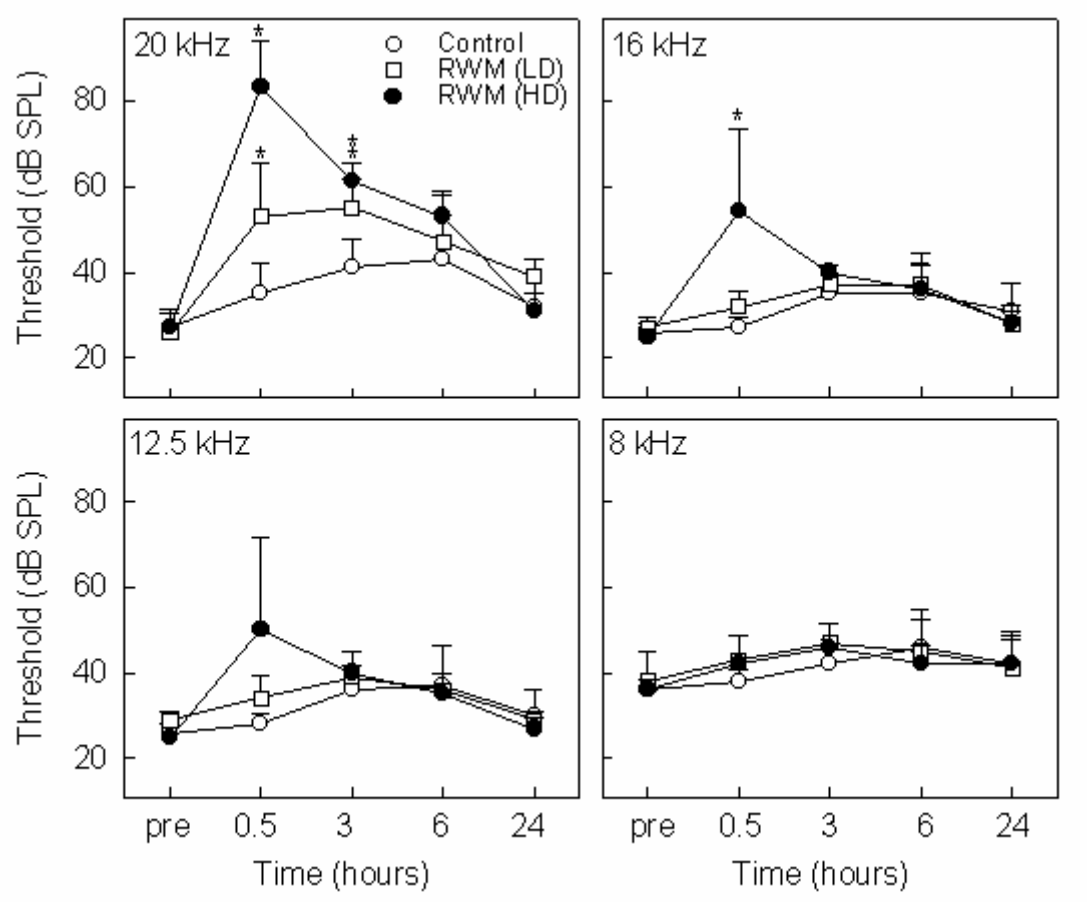


Fig. 18. ABR threshold (dB SPL) with time at 20, 16, 12.5 and 8 kHz. ABR threshold was affected most at 20 kHz and decreased with frequencies. At 8 kHz the ABR threshold was almost unaffect. The ABR threshold shifted most at 30 min and recovered partially at 3 and 6 h and returned to normal level at 24 h, with more effect on the HD group compared with LD group. In the control, there was only very slightly change in the ABR threshold.

*: $p<0.05$: statistically significant threshold shift compared with the control group.

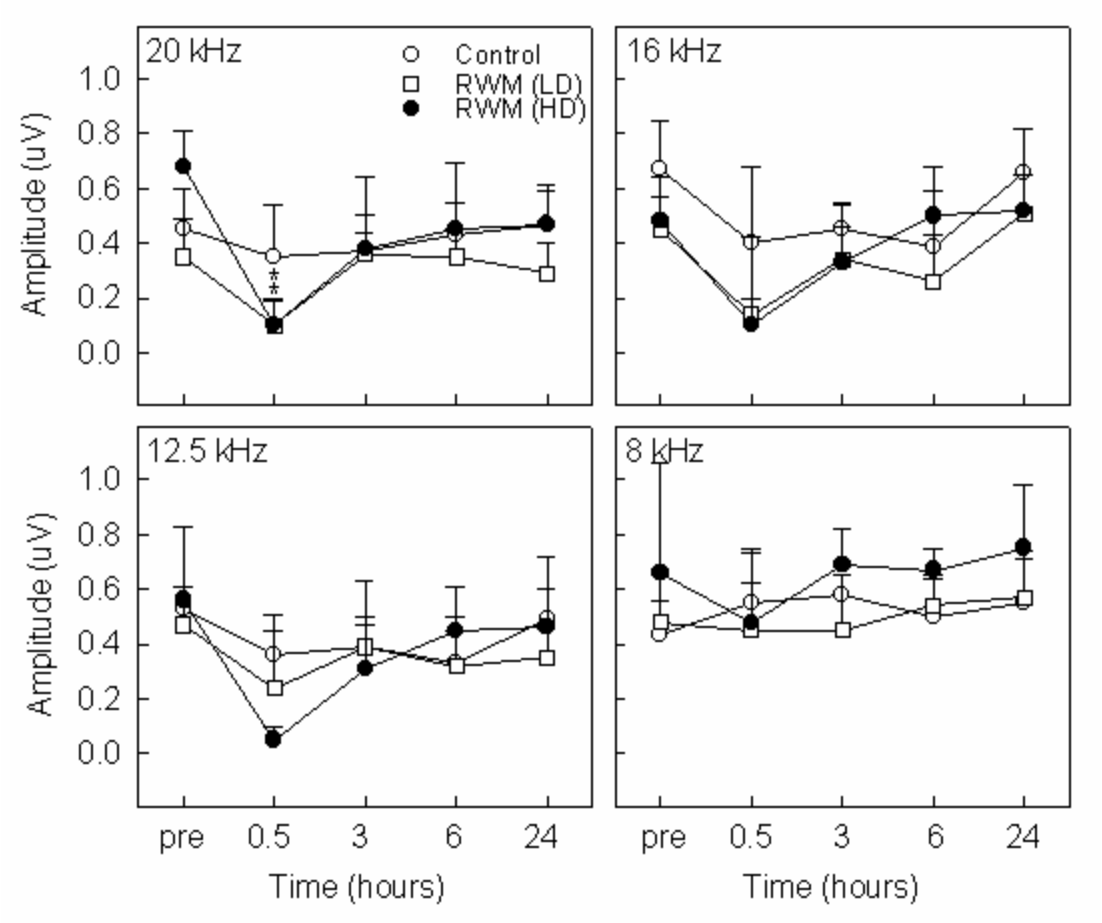


Fig. 19. Wave I amplitude (μV) at 90 dB with time at 20, 16, 12.5 and 8 kHz. The amplitude was decreased most at 20 kHz with less effect on lower frequencies. At The wave I amplitude was decreased most at 30 min and recovered partially at 3 and 6 h and returned to normal level at 24 h.

*: $p<0.05$: statistically significant amplitude decrease compared with the control group.

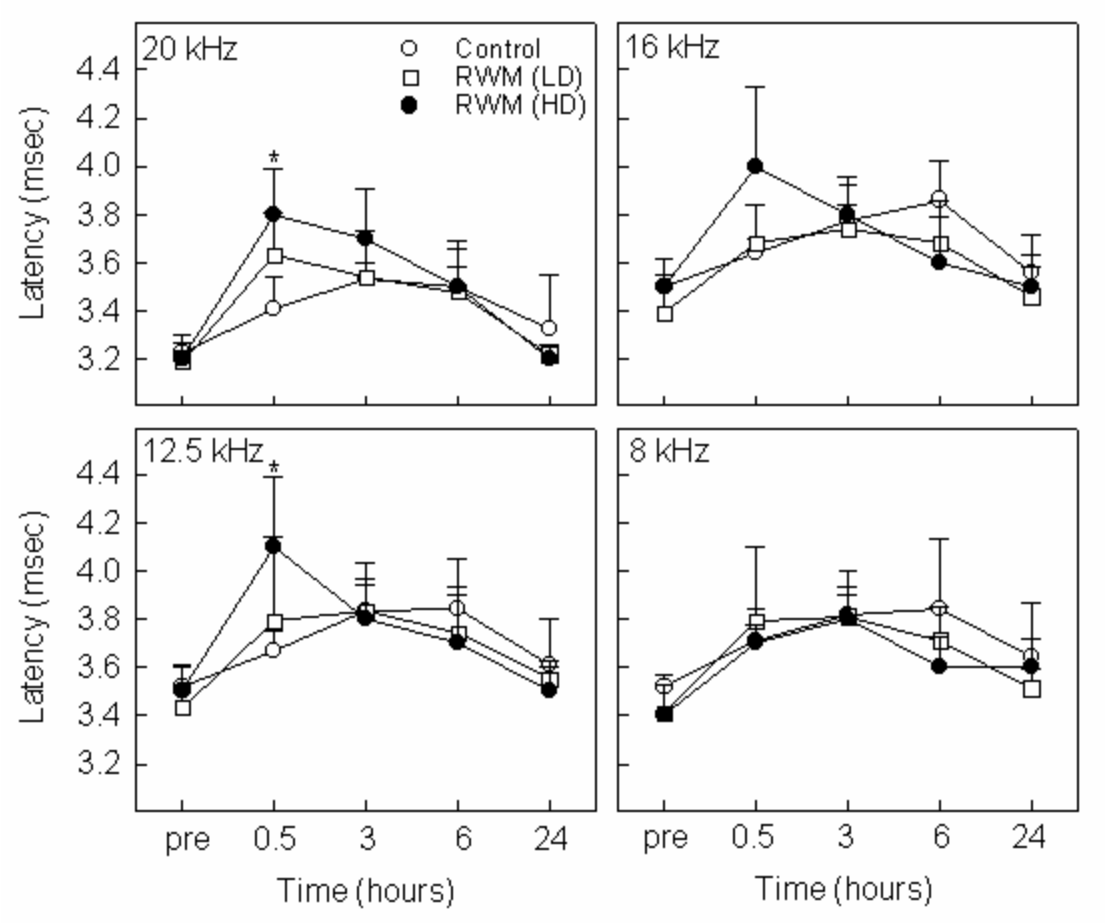


Fig. 20. Wave I latency (msec) at 90 dB with time at 20, 16, 12.5 and 8 kHz. The latencies were prolongated most at 20 kHz in the HD group, and there was little change at 16, 12 and 4 kHz in the LD and control group. The latency was affected most at 30 min and recovered partially at 3 and 6 h and returned to normal level at 24 h.

*: $p<0.05$: statistically significant latency prolongation compared with the control group.

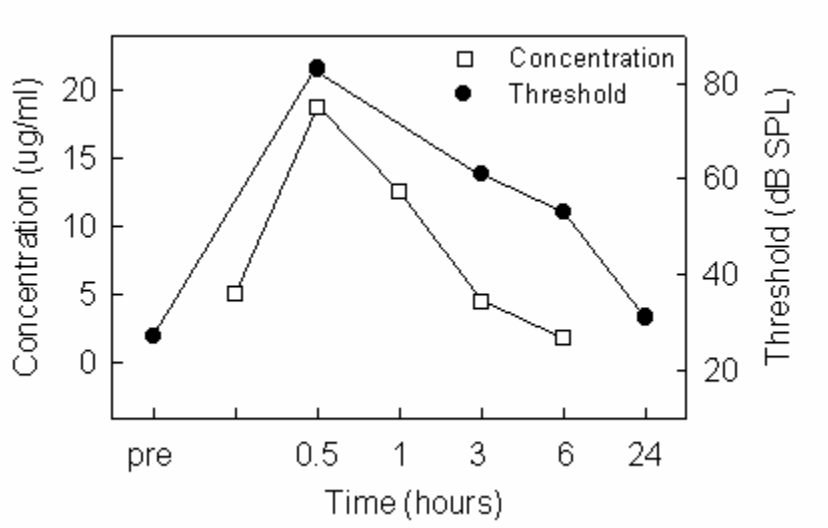


Fig. 21. Perilymph caroverine mean concentrations and thresholds at 20 kHz with time following high dose local application. At 30 min following application, the perilymph caroverine concentration reached peak value, and the ABR thresholds were shift most. At 3 and 6 h, the caroverine concentration in the perilymph decreased and the ABR threshold recovered partially.

The wave I of the ABR response comes from the activity of the eighth nerve, which innervates the cochlea region. To further explore the cochlea functional effects of local caroverine applications, the amplitude and latency of wave I were analyzed at 90 dB at all 4 frequencies (Fig. 19, 20). A statistically significant decrease in the wave I amplitude appeared at 20 kHz at 30 min in both the LD and HD groups compared to the control group ($p = 0.0004$, and $p = 0.026$, respectively). The wave I amplitude in the LD group showed less reduction than in the HD group. The amplitude partially recovered at 3 and 6 h and had returned to normal levels at 24 h following application.

The wave I latencies in the HD group were more severely changed than those in the LD group at all 4 frequencies (Fig. 20). When compared with the control group, significant latency prolongations were observed at 20 and 12.5 kHz at 30 min in the HD group ($p = 0.007$, and $p = 0.030$, respectively). The latencies in all 3 groups recovered partially at 3 and 6 h and recovered at 24 h after application.

The guinea pig's behaviors were observed when it woke up throughout the experiment. Vestibular disorders, such as imbalance, locomotor hyperactivity, ataxia and stereotypic head-movement were not seen.

Protective effect on NIHL

The protection of auditory function with caroverine was tested in the LD, HD, and control groups with RWM applications immediately prior to noise. The pre-exposure thresholds were shown in Fig. 22. The thresholds were around 20-30 dB at 20, 16, 12.5 and 4 kHz, and 35 dB at 8 kHz. There was no significant difference between control group, LD group and HD group. ABR threshold shifts, determined by the comparison of the post-exposure thresholds at regular time points with the pre-exposure thresholds, are plotted in Fig. 23. All 3 groups showed threshold shifts ranging from 50 to 70 dB across frequencies at 1.5, 3 and 6 h after noise exposure, irrespective of whether this was from control or caroverine treatment groups. At 24 h after noise exposure, the control group showed a recovery of around 20 dB at all frequencies tested. For the caroverine groups, however, the recovery was much more pronounced. At 24 h following caroverine application, the HD group showed

a 40-50 dB threshold recovery at 20, 16, 12.5 and 4 kHz, and about 30 dB recovery at 8 kHz. The threshold recovery was significantly larger than in the control group at all tested frequencies ($p<0.05$). In the LD group, the recovery was smaller than that in the HD group, but was still significant compared to the control group at 24 h after caroverine administration at the 2 highest frequencies (a 20-35 dB recovery at 20 and 16 kHz; $p = 0.0001$, and $p = 0.002$, respectively).

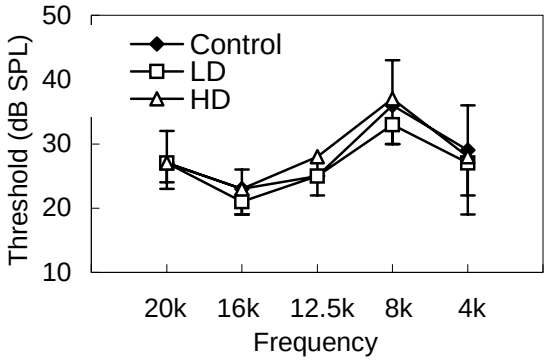


Fig. 22. Pre-exposure ABR threshold across frequencies tested. There was no significant difference among control group and LD and HD groups.

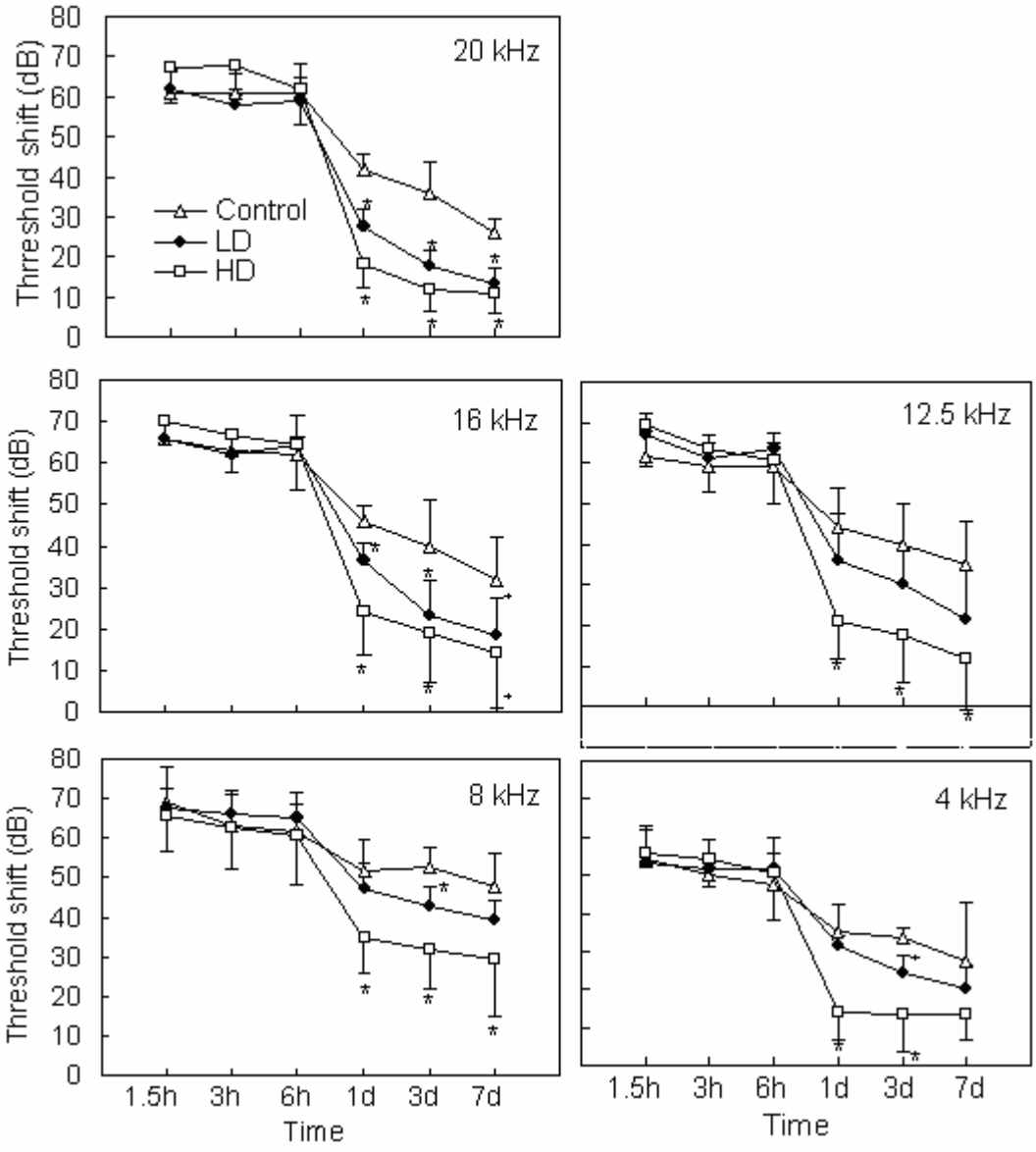


Fig. 23. ABR threshold shifts (mean ± SD) with time following noise exposure (Physiological saline or caroverine were applied onto the RWM 10 min before noise). At the first 6 h following noise exposure, there was no significant difference between control group and experimental groups. At 1, 3 and 7 days, the caroverine group had significant ABR threshold recovery compared with control group. The recovery in the HD group was more pronounced than LD group, with high frequencies better than low frequencies.

*: $p<0.05$: statistically significant threshold shift compared with the control group.

Further recovery of threshold was monitored at 3 days and 1 week following RWM applications. In all 3 groups, threshold recovered around 5 dB at 3 days compared to that at 24 h and also nearly 5 dB at 1 week compared to that at 3 days. Significant difference in threshold shifts were still present at 20, 12.5, 16 and 8 kHz in the HD group compared to the control group at 3 days and 1 week after caroverine application, and at 4 kHz at 3 days. In the LD group, threshold shifts of significant difference compared with the control group were observed at 20, 16, 8 and 4 kHz at 3 days, and at 20 and 16 kHz at 1 week after RWM application.

The guinea pig's behaviors were observed in the first 6 h of experiment and once every following day after it woke up from the sedative condition. Vestibular disorders, such as imbalance, locomotor hyperactivity, ataxia and stereotypic head-movement were not seen.

Therapeutic effect on NIHL and time window

The therapeutic effect was tested by ABR measurement on the groups with RWM application either 1 h or 24 h after noise exposure. The effect of caroverine applied 1 h after noise is illustrated in Fig. 24. Half an hour after RWM applications (1.5 h after noise exposure), both control and caroverine groups showed 60-70 dB threshold shifts at 8-20 kHz and nearly 45 dB threshold shift at 4 kHz. The threshold shifts in the control group remained at 35-45 dB at 20, 16, 12.5 and 4 kHz and at 50 dB at 8 kHz, 24 h after RWM saline application. However, in the caroverine group the threshold shifts decreased to 15-20 dB at 20, 16, 12.5 and 4

kHz and to 35 dB at 8 kHz, 24 h after caroverine application. The threshold shifts in the caroverine group were significantly smaller at all tested frequencies when compared to those of the control group at 24 h, 3 days and one week after RWM administrations.

The effect of caroverine applied 24 h after noise is illustrated in Fig. 25. The threshold shifts in the control group at 0.5 h after RWM application (24.5 h after noise exposure) were 25-40 dB at all tested frequencies. However, in the caroverine group, the threshold shifts were 50-60 dB at 20, 16, 12.5 and 8 kHz and 25 dB at 4 kHz. The control groups had better ABR thresholds because the hearing had partially recovered at 24.5 h after noise exposure. While in the caroverine group, caroverine would increase the ABR threshold itself and thus resulted in around 30 dB higher threshold shift than in the control group. Twenty-four hours after RWM application (48 h after noise exposure), both control and caroverine groups showed 15-25 dB threshold shifts at 20, 16, 12.5 and 4 kHz and 40 dB at 8 kHz. No significant difference in threshold shift was found between the control and caroverine groups at 24 h, 3 days and 1 week after RWM application.

The guinea pig's behaviors were observed in the first 6 h following RWM applications and once every following day after it woke up from the sedative condition. Vestibular disorders, such as imbalance, locomotor hyperactivity, ataxia and stereotypic head-movement were not seen.

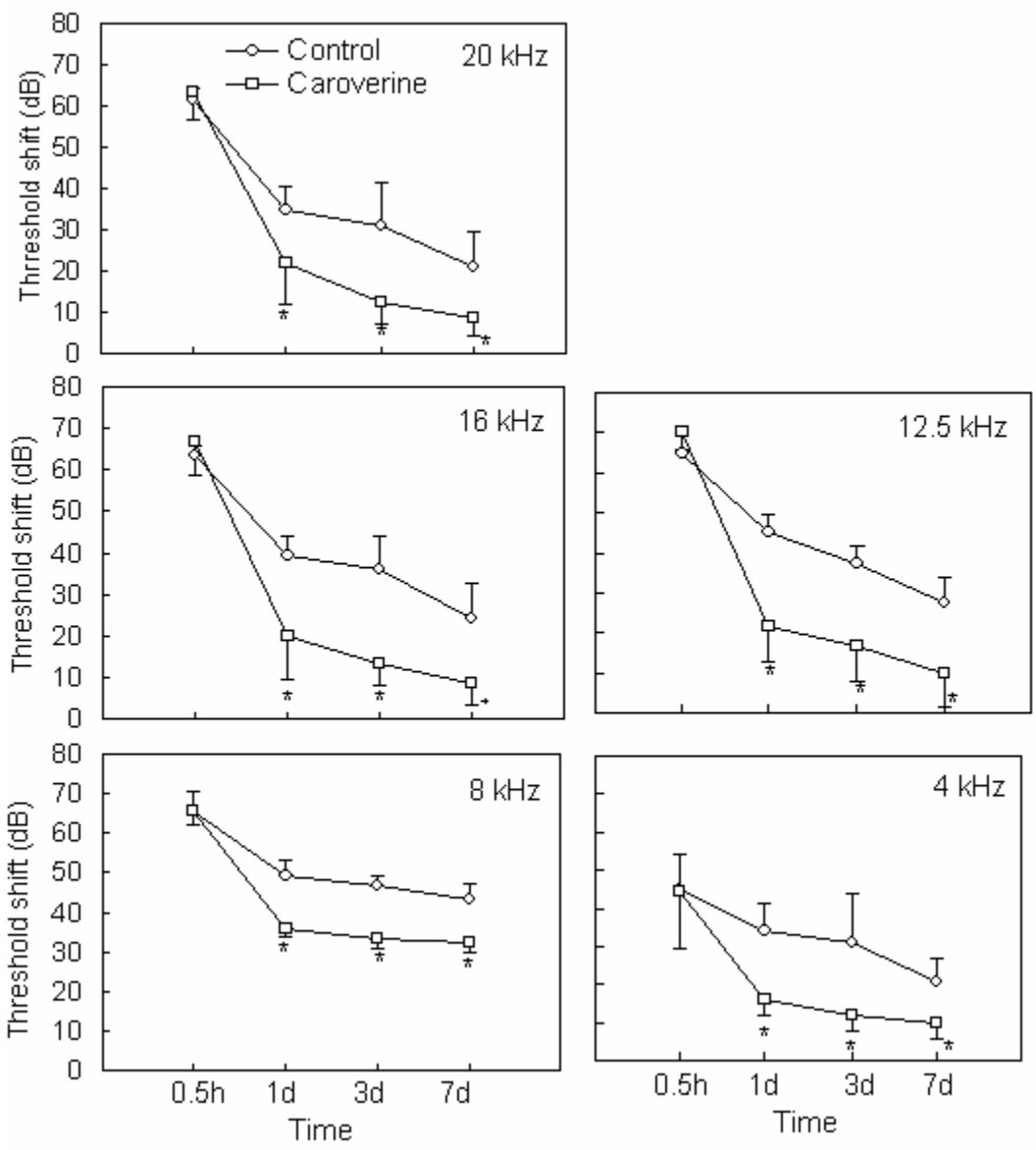


Fig. 24. ABR threshold shifts (mean ± SD) with time following RWM physiological saline or caroverine applications (1 h after noise exposure). At 0.5 h after RWM application, there was no significant difference in threshold shift between caroverine group and control g roup. At 1, 3 and 7 days after RWM administration, the recovery of ABR threshold in the caroverine group was of significant difference compared with control group across tested frequencies.

*: $p<0.05$: statistically significant threshold shift compared with the control group.

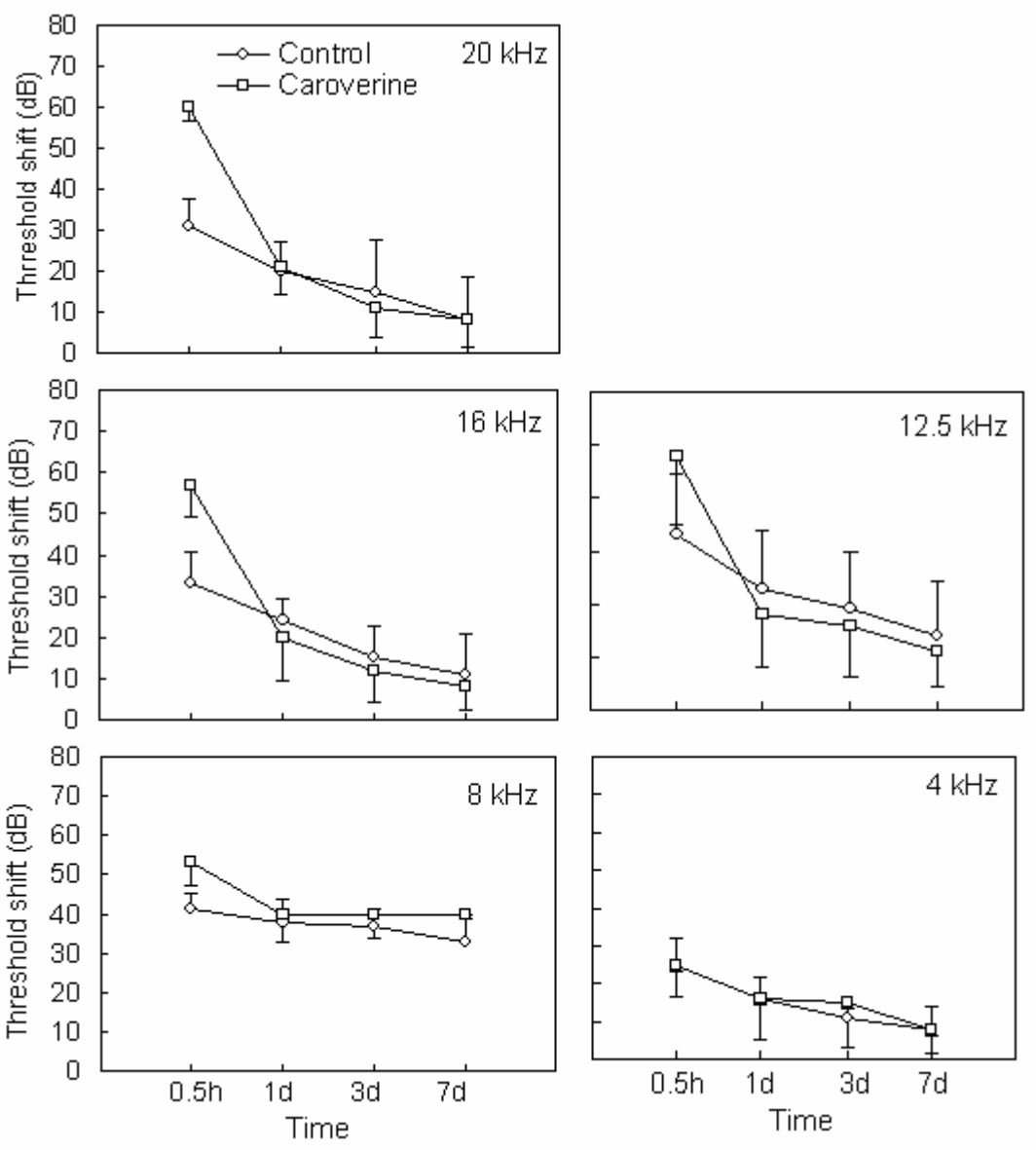


Fig. 25. ABR threshold shifts (mean ± SD) with time following RWM physiological saline or caroverine applications (24 h after noise exposure). At 0.5 h after RWM application, caroverine group had around 15-30 dB more threshold shift at 20, 16, 12 and 8 kHz compared with control group. This might be due to the recovery of hearing in the control group at 24 h after noise exposure and caroverine affected ABR in the experimental group. At 1, 3 and 7 days, there was no significant difference in ABR threshold shifts between caroverine group and control group.

DISCUSSION

Pharmacokinetics of caroverine in the inner ear and its effects on the auditory function following local RWM and systemic applications

There is growing interest in inner ear medication by local routes instead of systemic application, in order to achieve therapeutic drug levels in the inner ear while avoiding undesirable systemic side effects. Caroverine, as a glutamate receptor antagonist and an antioxidant in combination with calcium channel blocking activity, is a spasmolytic drug and also clinically used for the treatment of tinnitus, sudden hearing loss, speech discrimination disorders and other neurotoxic effects, such as ischemia/reperfusion, hypoglycemia, anoxia, hypoxia, shock and dementia (Denk et al., 1997; Saletu et al., 1996; Ehrenberger, 2002). However, the risk of inducing unwanted side effects appears in most glutamate receptor antagonists if given in large enough doses. Low doses are associated with altered sensory perception, dysphoria, hypertension, nystagmus and disorientation, with progression to agitation, paranoia, hallucinations, severe motor retardation and ultimately catatonia at higher doses (Muir and Lees, 1995). These potentially detrimental adverse effects obviously limit their clinical use for treatment of inner ear diseases by systemic administration. Thus, it is important to find alternative ways for the drug administration.

Delivery of agents into the inner ear via the RWM is being increasingly used clinically. For instance, this approach has been utilized for the delivery of steroids and gentamicin to the inner ear in the treatment of autoimmune diseases, sensorineural hearing loss, tinnitus and Ménière's disease (Silverstein et al., 1996, 1999; Blackley, 1997; Parnes et al., 1999; Hoffer et al., 2001; Schoendorf et al., 2001). Practically, this can be done by insertion of a microcatheter system or a MicroWick directly onto the RWM, or by using an implantable drug delivery system, which results in a more controlled application of the drugs (Lehner et al., 1997; Silverstein, 1999; Schoendorf et al., 2001). An alternative method is to instill the drugs via the tympanic membrane directly into the middle ear using gelfoam on the RWM as a form of continuous-release vehicle, which allows for prolonged drug perfusion of the labyrinth. This method is a relatively simple and effective procedure that has been used in both experimental and clinical studies (Silverstein et al., 1996, 1999). The disadvantages of the method are that it is only one single dose application and can not apply the drug repeatedly.

The present study showed that by local applications of caroverine onto the RWM with gelfoam, the perilymph concentrations quickly reached high peak values at 30 min, followed by a relatively fast decrease over time. The perilymph peak values in the LD and HD RWM groups were almost 20 and 80 times higher than the peak value reached in the IV group. As expected, the high dose produced higher perilymph levels than the low dose, which suggests that the absorption of caroverine through the RWM is a dose-dependent process. The perilymph

caroverine concentration fell more slowly in the animals given the drug directly onto the RWM than in those animals given a much higher dose systemically. In the perilymph, caroverine might be removed not only by passive diffusion to endolymph or to the CSF through the cochlear aqueduct, but also by active elimination such as blood flow and lymphatic flow (Hibi et al., 2001). It is possible that the elimination of caroverine is faster than what was shown in the two local groups. The maintenance of caroverine concentration in the perilymph in local groups is most likely due to the continuous absorption of caroverine from the gelfoam through the RWM. It is well known that the RWM is permeable to various drugs and substances placed in the round window niche area. These include antibiotics, antiseptics, arachidonic acid metabolites, local anesthetics, toxins and albumin (Goycoolea and Lundman, 1997), showing that not only small molecules but also macromolecules can pass through the RWM. Caroverine is a low-molecular-weight substance (molecular weight of caroverine hydrochloride = 420) and should pass through the RWM quite freely. The concentrations of caroverine in the perilymph after local applications were considerably higher than those found following systemic application. These results clearly illustrated the permeability of the RWM to caroverine. Consequently, high concentrations of caroverine can be attained in the perilymph by application of a small amount of the drug on the RWM.

After systemic administration, the concentrations of caroverine in the plasma were consistently higher than those in the CSF and perilymph. This can be attributed to the existences of both blood-brain and blood-labyrinth barriers. The perilymph concentrations seemed to be higher than CSF concentrations, but the difference was not statistically significant. This may reflect the differences between the blood-brain barrier and blood-labyrinth barrier, or the properties of the communication between the CSF and perilymph through the cochlear aqueduct. Another important observation, although not entirely unexpected, was that local caroverine applications resulted in much lower drug concentrations in the plasma and CSF as compared to systemic administration. For example, the plasma and CSF caroverine peak values in the IV group were about 12 and 6 times the peak values in the HD group, respectively. A less systemic adverse effect may be expected with the lower caroverine concentrations in plasma and CSF. This is in part related to the dose. The total volume of the perilymph is only around 15.9 mm^3 in the guinea pig cochlea (Shinomori et al., 2001). Therein lies the major advantage of the RWM administration, which is the ability to achieve a high local drug concentration without high blood and CSF levels.

The ideal concentration of caroverine in the perilymph for the treatment of inner ear diseases, such as tinnitus, remains unknown. In this study, the intravenous dose used in guinea pigs was the same as that used clinically in the treatment of tinnitus. Assuming similar pharmacokinetics in guinea pigs and humans, one may presume the therapeutic concentration of caroverine to be around 0.2--0.3 μg/ml in the

perilymph, which was the peak caroverine concentration in the perilymph after systemic administration in the present study. The two dosages for RWM applications were chosen somewhat arbitrarily. However, the main purpose of the study was not to establish a therapeutic window but to relate the auditory effects to caroverine concentrations in the perilymph. The effects on hearing threshold were tested by measuring the ABR to sound stimuli following local caroverine applications. The perilymph caroverine concentration is expected to be higher in the basal, high frequency region of the cochlea, being closer to the RWM. Indeed, the maximum changes in the ABR threshold, latency and amplitude occurred at 20 kHz at 30 min after applications in both local administration groups, and the changes were all statistically significant. The ABR was less affected at the frequencies of 16, 12.5 and 8 kHz, most likely due to the lower caroverine concentrations at positions further from the RWM. The ABR thresholds recovered partially at 3 and 6 h and were completely back to normal levels 24 h after administration. The two local dosages caused transient, but reversible hearing dysfunction. The hearing dysfunction may be due to the reason that caroverine binds to both NMDA and AMPA receptors, thus blocking the activity of the neurotransmitter glutamate and consequently the transduction of the sound. As the transient dysfunction is dose related, one would expect it to be negligible at the assumed therapeutic concentration, which is much lower than the perilymph concentration after RWM application in the study. The slight hearing impairment seen in the control group was most likely due to the weight of gelfoam and saline on the RWM, surgical stress and possibly an altered ionic balance as sodium and chloride ions will enter

the perilymph when saline is applied on the RWM (Molinari, 1972; Colletti et al., 1986; Hisashi et al., 1999).

By combining the pharmacokinetic observations with the changes seen in the ABR thresholds in the HD group, it is clearly demonstrated that the ABR effect was related to the concentration of caroverine in the perilymph. Fig. 21 manifests both the perilymph caroverine concentrations and the ABR thresholds (at 20 kHz) as functions of time following a high dose RWM administration. At 30 min, when perilymph caroverine reached its peak value, the ABR threshold shift was also the greatest. At 3 and 6 h, when the perilymph caroverine concentration decreased markedly, the ABR threshold accordingly recovered partially.

Further studies are necessary to find out the ideal dose and administration paradigm. The study of the effect following local applications of caroverine on the RWM in the treatment of excitotoxicity-related inner ear diseases, such as noise-induced hearing loss, can be carried out on the animal models. This information will be useful for the establishment and formulation of the local application method in the clinic in the future.

Protection of auditory function against noise trauma

This part of study demonstrated that local caroverine administrations directly onto the RWM immediately prior to noise exposure produced significant protection of auditory function against noise. The protective effect was dose-dependent, with

greater effect in the HD group than in the LD group. The significant protection, as measured by using the ABR threshold, was found at 24 h, 3 days and 1 week after noise exposure at all tested frequencies (4-20 kHz). These results support the notion that pharmacological protection of cochlear function has a promising potential for the prevention of noise-induced hearing impairment.

Glutamate receptor antagonists, such as MK-801 and kynurenic acid, have been shown to be effective in the protection of neuronal dendrite damage beneath the IHCs against noise trauma and consequently to preserve hearing (Puel et al., 1998; Duan et al., 2000; Chen et al., 2001). During noise exposure, it appears that AMPA receptors are activated by low-to-moderate stimulus intensities, whereas NMDA receptors are activated by high-intensity sounds (Felix and Ehrenberger, 1991). In our experiments, noise exposure at 110 dB SPL would have activated both NMDA and AMPA receptors. Our results suggest that the NMDA and AMPA receptors on the afferent dendrites are being blocked by their antagonist caroverine, thus the glutamate excitotoxicity due to noise exposure is limited and the cochlear functional damage is prevented.

Another possible explanation to the protection of auditory function against noise trauma may be based on the antioxidant activity of caroverine. Glutamatergic neurotoxicity is partially caused by the overproduction of ROS and following membrane damage by lipid peroxidation (Azbill et al., 1997; Simonian and Coyle, 1996). The accumulating evidences show that ROS play an important role in NIHL.

Ohlemiller et al. (1999) demonstrated that ROS increased nearly 4-folds in the cochlea during the first 1-2 h after intense noise exposure and did not decrease over that time. ROS were shown to affect the isolated OHC morphology or impair cochlear function after perilymphatic space infusion (Cleric et al., 1995; Cleric and Yang, 1996). A variety of antioxidants such as superoxide dismutase-polythylene glycol and allopurinol could protect cochlear damage when applied prior to noise exposure (Seidman et al., 1993). Caroverine is a strong antioxidant as demonstrated recently by Udilova et al. (2003). They found that the antioxidant activity of caroverine was not only the potent removal of $OH^{\bullet}$-radicals but also the ability to interfere into $OH^{\bullet}$-radical generation and thus to inhibit the establishment of oxidative stress. Consequently, it is reasonable to expect caroverine to prevent ROS toxicity to the cochlea after noise exposure.

The protection of cochlea from noise trauma might also due to the calcium channel blocking activity of caroverine. Four types of voltage-activated calcium channels have been identified in various cell membranes: N, T, P and L type calcium channels (Spedding and Paoletti, 1992; McCleskey, 1994). The L-type channel (slow inactivation and susceptibility to the dihydropyridine class of blockers, such as nimodipine) has been demonstrated to be present in the mammalian inner ear tissues with molecular biological studies (Green et al., 1996; Kollmar et al. 1997 a, b). It has been found that the L-type channel is the only channel type present in the chick cochlear hair cells (Zidanic and Fuchs 1995), however, the presence of at least of one other type (possibly N-type) in addition to L-type channels has been

suggested (Prigioni at al., 1992; Su et al., 1995). The calcium channels have been shown to play important roles in controlling neurotransmitter release from the hair cells which are important in regulating excitation of the auditory nerve fibers (Robertson and Johnstone 1979; Issa and Hudspeth 1994; Tucker and Fettiplace 1995; Zidanic and Fuchs 1995; Kollmar et al. 1997a, b; Martinez-Dunst and Fuchs 1997). In addition, voltage-gated calcium channels can regulate the conductance of the basolateral wall of the hair cells mainly through the action of calcium-activated potassium channels (Issa and Hudspeth 1994; Art and Fettiplace 1995; Dulon et al. 1998) and may also contribute to the regulation of other calcium-dependent aspects of hair cell fuction, including adaptation and slow contractile processes (Zenner et al. 1985; Ulfendahl 1987; Assad and Corey 1992). In the guinea pig, it has been demonstrated that L-type calcium channels are involved in the various aspects of cochlear response to sound and in the transmitter release from the IHCs (Bobbin et al., 1990; Zhang et al., 1999). Intense noise exposure might disturb the hair cell metabolism, especially the calcium-regulatory processes. The altered calcium concentration in the inner hair cells might increase the neurotransmitter release and induce excitotoxicity. Thus, calcium antagonists would be able to protect the cochlea from noise trauma. In fact, certain calcium channel blockers, such as diltiazem, have been shown to protect the inner and outer hair cells from intense noise exposure in the guinea pig (Mann et al., 1987; Maurer et al., 1999; Heinrich et al., 1999). However, some other studies showed that calcium channel blockers, for instance, nimodipine and diltiazem, could not provide any benefit effect against noise trauma in the gerbil, mouse or human (Maurer et al., 1995; Boettcher, 1996;

Ison et al., 1997; Boettcher et al., 1998). This may be due to the different dosing protocols or different expression of calcium channels in the cochleae in different species. Caroverine has unspecific calcium channel blocking activity (Hornykeiwicz, et al., 1963). The protective effect of caroverine against noise trauma might partly be contributed to its blocking of calcium channels in the hair cells, preventing calcium influx into the hair cells. This might prevent the damage of excessive calcium to the hair cells and also reduced the over-release of excitatory neurotransmitter from inner hair cells and thus decreased the excitotoxicity.

Our previous study demonstrated that caroverine readily penetrated the RWM in the guinea pig (Chen et al., 2003). Caroverine concentration in the perilymph reached its peak value at 30 min after both low and high dose local applications with gelfoam. In the LD group, caroverine became undetectable in the perilymph within 6 h, while in the HD group caroverine still remained at a high level at 6 h after RWM application. The effect on hearing was mainly seen at the higher frequencies (i.e., closer to the round window). At 30 min there was a 56 dB threshold shift at 20 kHz, which recovered partially at 3 and 6 h. Thresholds became normal at 24 h. The present study shows that caroverine can effectively protect the cochlear function against noise trauma when applied immediately prior to noise exposure. Within the first 6 h after RWM applications, there was no significant difference in threshold shift between the LD or HD group and control group. This is most likely due to high concentration of caroverine in the perilymph binding to the glutamate receptors, thus blocking the effect of the neurotransmitter (glutamate) and the sound

transduction. However, at 24 h after caroverine application, there was a significant decrease in noise-induced threshold shifts. In addition, the HD group, with a higher caroverine concentration in the perilymph, as shown previously (Chen et al., 2003), maintained greater protective effect as compared to the LD group. The protection was still manifest 1 week after caroverine application.

The frequency effect observed in this study is of particular interest. The protective effect is of the most significance at 20 kHz. This could be attributed to the higher concentration of caroverine in the basal, high frequency region of the cochlea, being closer to the RWM. After noise exposure, there was a more severe threshold elevation at 8 kHz, which is compatible to the well-known phenomenon of 'half-octave shift' in a damage cochlea (Davis et al., 1950; Greenwood, 1993). According to the half-octave shift, the most vulnerable frequency is around 9 kHz when exposed to 6.3 kHz band noise.

However, the auditory threshold did not recover completely to normal level even at 1 week after caroverine application in the HD group. There were still 10-15 dB threshold shifts at 20, 16, 12.5 and 4 kHz, and 30 dB at 8 kHz. Glutamate receptor antagonists other than caroverine, including NMDA or AMPA receptor antagonists, only partially protect the cochlear function against auditory impairment (Liu and Fechter, 1995; Puel et al., 1998; Duan et al., 2000). This may be partially due to the incomplete reestablishment of the hair cell neural synaptic contacts. That glutamate receptor antagonists do not completely protect against NIHL reflects the fact that

other mechanisms are also involved. Two main mechanisms are proposed to be involved in NIHL: mechanical damage, which is permanent and irreversible, and metabolic alterations. Damage to the OHC is thought to be especially important for NIHL based on the histological studies using surface preparation (Ryan and Dallos, 1975; Stebbins et al., 1979; Hamernik et al., 1989). Glutamate receptors are demonstrated to be present mainly on the spiral ganglion cell and sparely on the IHCs and be absent on the OHCs, although glutamate receptor expressions are transiently presented on the OHCs in the developing ear (Safieddine and Bybalin, 1992; Niedzielski and Wenthold, 1995; Usami et al., 1995; Matsubara et al., 1996).

The cellular and molecular mechanisms underlying hearing loss are not well known. The impairment of the inner ear after noise trauma may involve glutamate-mediated excitotoxicity, oxidative stress, apoptosis of hair cells and auditory neurons (Aarnisalo, et al., 2000; Hu et al., 2000; Hu et al., 2002; Nicotera, et al., 2003; Wang at al., 2002; Wang et al., 2003), unregulated calpain proteolysis (Stracher, 1999; Shulman, 1998; Lefebvre et al., 2002), and the decrease of the microcirculation of the cochlea (Quirk and Seidman, 1995; Seidman, 1999). Different agents have been applied to interfere these pathways for the protection of cochlea against noise trauma. Wang et al. demonstrated that the anti-apoptosis agent riluzole could prevent the noise-induced permanent hearing loss when perfused into the cochlea via an osmotic minipump in the guinea pig (Wang et al., 2002). The calpain inhibitor leupeptin has been shown to significantly protect the cochlea from noise trauma (Salvi et al., 1998; Wang et al., 1999). Lamm and Arnold found that

the blood flow promoting drugs, such as hydroxyethyl starch, pentoxifylline and ginkgo biloba, could compensate cochlear ischemia and reduce noise-induced hearing loss (Lamm and Arnold, 2000). Neurotrophins, including nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3) and glial cell line-derived neurotrophic factor (GDNF), are known to play a role in the survival of injured auditory neurons (Altschuler et al., 1999; Ylikoski et al., 1998; Staecker et al., 1996). The protective effects of the neurotrophins against noise trauma have been widely studied. Numerous reports have shown that neurotrophins, such as NT-3, BDNF, GDNF and NGF, could protect the auditory neurons and hair cells from noise trauma (Keithley et al., 1998; Duan et al., 2000; Shoji et al., 2000 a, b). Since these agents protect the cochlea from noise-induced hearing loss by different mechanisms, it might be more promising to combine caroverine with these agents to protect the cochlea from noise trauma.

In conclusion, the result of this part of study demonstrates that caroverine can significantly protect the cochlea from noise trauma when applied onto the RWM immediately prior to noise exposure. The protective effect of caroverine, an NMDA and AMPA receptor antagonist together with antioxidant and calcium channel blocking activities, against noise trauma supports the notion that the excessive release of glutamate from the IHCs and the consequent excessive ROS production plays an important role in the pathophysiology of NIHL. Thus, pharmacological protection of the cochlea against noise is possible and may be of great clinical potential.

Therapeutic effect and time window on noise trauma

This part of study shows that local RWM administration of caroverine 1 h after noise exposure significantly reduces the damage caused to cochlear function after noise trauma. The significant therapeutic effect was found at 1 day after noise exposure and was still present at 1 week during the functional recovery. In contrast, treatment with caroverine 24 h after noise exposure failed to achieve any functional protection during the same time period.

In the previous study (Chen et al., 2003), high concentration of caroverine was detected in the perilymph during the first 6 h following RWM application with the same protocol and dose as in the present study, and the effects on ABR threshold were related to the concentration of caroverine in the perilymph. In the present results, there was no significant decrease in threshold shifts in the caroverine group compared with control group at 0.5 h after RWM application (1.5 h after noise exposure). The hearing loss in the control group was caused mainly by noise and partially by surgery which was demonstrated in the previous study (Chen et al., 2003), while the threshold shifts in caroverine group might be induced by the blockage of neurotransmitter glutamate receptors with high concentration of caroverine, and by noise and surgical operation.

Interestingly, significant improvement of auditory function was found at 24 h, 3 days and 1 week after caroverine application.

The time at which the treatment is begun is a critical issue. In the central nervous system, glutamate is released early after traumatic injury, and early treatment is necessary (McIntosh, 1994). In animal models of cerebral ischemia, NMDA receptor antagonists were effective against ischemia injury only when administered before or shortly after the ischemia insult (Meldrum, 1990). Talampanel, AMPA receptor antagonist, is shown to significantly attenuate neuronal damage when administrated 30 min, but not 3 h, after traumatic brain injury in rats (Belayev et al., 2001). However, the AMPA/kainate receptor antagonist NBQX is effective in reducing damage in rats subjected to global ischemia even administered several hours after the ischemic insult (Sheardown et al., 1990; Li and Buchan, 1993). During high-level noise exposure, significant increase of glutamate was detected in the guinea pig's cochlea by using microdialysis (Jäger et al., 2001). But the duration of increased glutamate level in the cochlea following noise exposure remains unknown. In the present study, early treatment with caroverine at 1 h after noise trauma led to significant reduction of auditory functional impairment. However, when caroverine treatment began 24 h after noise trauma, the protective effect on hearing was lost. Our results may imply that excessive glutamate is still present in the cochlea and damages the hearing even 1 h after noise exposure.

It has been demonstrated that AMPA/kainate receptors are activated by low-to-moderate intensity sound, while NMDA receptors are activated by high-intensity stimulus (Felix and Ehrenberger, 1991). In this study, noise at 110 dB SPL would have activated both NMDA and AMPA/kainate receptors. It is suggested that

NMDA-receptor activation under excessive glutamate is mainly responsible for the initial disturbance of neuronal ion homeostasis, while AMPA/kainate receptors contribute to the development of neuronal damage at a stage when NMDA receptors begin to play a less prominent role (Prehn et al., 1995). Moreover, the molecular study on the expression of AMPA receptor subunits (GluR1, GluR2, GluR3, and GluR4) suggested that when GluR2 is coexpressed with one or more of the other subunits, the AMPA receptor has very low permeability to Ca^{2+}. But when GluR2 is absent, the AMPA receptor has substantial Ca^{2+} permeability (Meguro et al, 1992; Belayev et al, 2001). The observation of a relative loss of mRNA for GluR2 in postischemic CA1 zone implicated a potential mechanism of an increase in Ca^{2+} permeability of AMPA receptors (Pellegrini-Giampietro et al, 1992). The increased Ca^{2+}-permeable AMPA receptor might account for the susceptibility of postischemic neuron and the ability of delayed AMPA receptor antagonist for prevention of Ca^{2+} influx (Andine et al, 1992). It was suggested that the ischemia-induced damage in the cochlea was via the activation of excitotoxicity which occurred in the NIHL as well (Pujol et al., 1993). When applied 1 h after noise exposure, caroverine, an NMDA and AMPA receptor antagonist, would block both the two receptors and thus limit the excitotoxicity and preserve hearing.

The mechanism by which caroverine could attenuate auditory impairment when applied 1 h, but not 24 h, after noise exposure is not fully elucidated. One possible explanation might be that the auditory functional impairment was caused by both metabolic and mechanic damages due to noise trauma. The process of metabolic

change might still continue at 1 h after noise exposure via glutamate release and the excessive glutamate could not be eliminated at this time period, and the consequently increased ROS production might damage the cochlea. It was demonstrated that ROS concentration increased almost 4-folds in guinea pigs' cochlea during the first 1-2 h after intense noise exposure and did not decrease over that time (Ohlemiller et al., 1999). The production of ROS plays an important role in NIHL as discussed previously. However, the metabolic process might have ceased at 24 h after noise trauma. If so, caroverine will be able to block both NMDA and AMPA receptors, and its potent antioxidant activity makes it possible to inhibit the establishment of oxidative stress and to scavenge toxic ROS, thus leading to the rescue of the hearing.

The explanation for no significant improvement of cochlear function following caroverine administration 24 h after noise might also be suggested by studies on the expression of NMDA receptor after noise trauma. Indeed, *in situ* hybridization experiments revealed the expression of mRNA coding for NR1 subunit of NMDA in the spiral ganglion neurons increased 24 h after excitotoxic insult (Puel et al., 1995). This enhanced expression decreased slightly 2 days after exposure and returned to normal value at 3 days (Puel et al., 1995). In another study, chronic application of NMDA receptor antagonist during functional recovery after excitotoxicity delayed the regrowth of neurites and the restoration of hearing (Felix and Ehrenberger, 1991). These findings suggest that glutamate plays a neurotrophic role via activation of NMDA receptors in the post-traumatic cochlea. When

caroverine was applied 1 h after noise, its beneficial effect exceeded harmful effect on cochlear function, so the hearing was significantly preserved. But when the drug was applied 24 h after noise, its beneficial effect decreased and the hearing recovery was retarded.

In summary, this part of study demonstrated that acute treatment with caroverine is beneficial in limiting the auditory functional impairment after noise trauma. But the therapeutic window is narrow.

CONCLUSIONS

The present studies demonstrated that the RWM is permeable to caroverine, an NMDA and AMPA receptor antagonist together with antioxidant activity. Local application of caroverine with gelfoam directly onto the RWM is both safe and more effective. Administration of caroverine onto the RWM immediately prior to noise exposure can significantly protect the auditory function against noise. Caroverine can significantly rescue the hearing when applied 1 h, but not 24 h, onto the RWM after noise exposure. These results support the notion that the excessive release of glutamate from the inner hair cells and the consequent production of ROS play important roles in the pathophysiology of noise-induced hearing loss. Thus, pharmacological protection of the cochlea against noise is possible and may be of clinical potential.

FUTURE PERSPECTIVES

At present no drug has been approved for the local administration to the round window membrane for otoprotection or treatment of the inner ear diseases in human. For transfer of this RWM application therapeutic strategy into controlled clinical trials it is necessary to undergo preclinical studies to establish dose-effect relationships for both therapeutic and toxic effects (Spandow et al., 1988; Shirwany et al., 1998). Direct study of inner ear pharmacokinetic profiles following RWM application is not possible in human because human inner ear fluids can not be safely sampled without damage of the ear. The alternative is to predict drug levels in the inner ear in human from the results obtained in the animal experiments. Experimental animal study on the perilymph drug concentration after RWM application is important for the advancement of this therapeutic strategy. One important issue is that drugs applied locally onto the RWM are not equally distributed throughout the inner ear, which has been demonstrated by other studies (Stover et al., 1999; Salt and Ma, 2001) as well as our findings. The distribution along the length of the cochlea is dominated by the rate of diffusion of the drug with faster for small molecules and slower for large moleculars relative to the rate of clearance of the drug from the scala. Technically, it would not be possible to achieve a uniform drug distribution along the perilymphatic spaces using the RWM application approach. Therefore, drugs with different molecular sizes might not be equally distributed along the inner ear space even if they have the same average perilymph drug concentration. Thus every drug will have its own dose-effect relationship when applied onto the RWM. Since the RWM administration has the

advantages of high drug concentration in the perilymph with little systemic distribution, a wide range of drugs, especially those with severe systemic side effects when administrated systemically, can be applied onto the RWM to study the permeability of RWM to these drugs and dose-effect relationship in the animal. We would also apply these drugs to the RWM of animal models with certain inner ear disorders to study the therapeutic effect of the drugs. For example, corticosteroids have been used for the treatment of idiopathic sudden sensorineural hearing loss, immune related hearing loss by systemic applications (IV or oral) based on its anti-inflammatory effect (McCabe, 1979; Moskowitz et al., 1984). But the side effects associated with prolonged high-dose courses of systemic corticosteroids can be devastating and even fatal. Alternatively, cortixosteroids might be applied to the RWM locally in the animal model. Its penetration through the RWM and safety and efficacy can be tested on the animal.

It has been proposed that glutamate-induced excitotoxicity and oxidative stress are involved not only in noise-induced hearing loss, but also in other inner ear disorders, such as anoxia (Pujol et al., 1992; Puel et al., 1994), age-related hearing loss (Pujol et al., 1990; Seidman, 2000; Seidman et al., 2000). With both antiglutamatergic and antioxidant activities, caroverine might be tested for its protection and treatment of anoxia and age-related hearing loss in the animal model. Relatively, little is known about the neurontransmission in human inner ear because of the difficulty in the access of the human specimen. Recently, Nordang et al. (2000) for the first time identified L-glutamate, NMDAR2B and the enzyme

glutamine synthetase immunomorphologically in the sections from heathly human inner ears. They found that glutamate, NR2B and glutamine synthetase were distributed in synaptic regions in a similar way as desribed in many other animal species (Bobbin, 1979; Eybalin and Altschuler, 1990; Eybalin 1993). It may be assumed that glutamate also acts as neurotransmitter in the human cochlea. The understanding of glutamate receptors in the human cochlea is a prerequisite for the pharmacotherapy of the inner ear disorders related to overstimulation of the afferent terminals. Since caroverine has been demonstrated to protect the cochlea from noise-induced overstimulation of the cochlear afferent nerve, it is reasonable that caroverine might be used for the clinical trial treatment of excitotoxicity-related inner ear disorders, such as noise-induced hearing loss and tinnitus.

REFERENCES

Aarnisalo AA, Pirvola U, Liang XQ, Miller J, Ylikoski J. Apoptosis in auditory brainstem neurons after a severe noise trauma of the organ of Corti: intracochlear GDNF treatment reduces the number of apoptotic cells. ORL J Otorhinolaryngol Relat Spec 2000; 62: 330-4.

d'Aldin C, Eybalin M, Puel JL, Charachon G, Ladrech S, Renard N, Pujol R. Synaptic connections and putative functions of the dopaminergic innervation of the guinea pig cochlea. Eur Arch Otorhinolaryngol 1995; 252: 270-4.

Altschuler RA, Sheridan CE, Horn JW, Wenthold RJ. Immunocytochemical localization of glutamate immunoreactivity in the guinea pig cochlea. Hear Res 1989; 42: 167-74.

Andine P, Jacobson I, Hagberg H. Enhanced calcium uptake by CA1 pyramidal cell dendrites in the postischemic phase despite subnormal evoked field potentials: excitatorey amino acid receptor dependency and relationship to neuronal damage. J Cereb Blood Flow Metab 1992; 12: 773-83.

Art JJ, Wu YC, Fettiplace R.The calcium-activated potassium channels of turtle hair cells. J Gen Physiol 1995; 105: 49-72.

Assad JA, Corey DP. An active motor model for adaptation by vertebrate hair cells. J Neurosci 1992; 12: 3291-309.

Avraham KB. Motors, channels and the sounds of silence. Nat Med 1997; 3: 608-9.

Azbill RD, Mu X, Bruce-Keller AJ, Mattson MP, Springer JE. Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. Brain Res 1997; 765: 283-90.

Belayev L, Alonso OF, Liu Y, Chappell AS, Zhao W, Ginsberg MD, Busto R. Talampanel, a novel noncompetitive AMPA antagonist, is neuroprotective after traumatic brain injury in rats. J Neurotrauma 2001; 18: 1031-8.

Berger H. Citation from "The auditory brainstem response" Ed. John T. Jacobson, Taylor & Francis, 1985, London. 1929.

Blackley BW. Clinical forum: A review of intratympanic therapy. Am J Otol 1997; 18: 520-26.

Bleakman D, Ballyk BA, Schoepp DD, Palmer AJ, Bath CP, Sharpe EF, Woolley ML, Bufton HR, Kamboj RK, Tarnawa I, Lodge D. Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors in vitro: stereospecificity and selectivity profiles. Neuropharmacol 1996; 35: 1689-702.

Bledsoe SC Jr, Bobbin RP, Thalmann R, Thalmann I. Stimulus-induced release of endogenous amino acids from skins containing the lateral-line organ in Xenopus laevis. Exp Brain Res 1980; 40: 97-101.

Bobbin RP. Glutamate and aspartate mimic the afferent transmitter in the cochlea. Exp Brain Res 1979; 34: 389-93.

Bobbin RP, Jastreboff PJ, Fallon M, Littman T. Nimodipine, an L-channel Ca2+ antagonist, reverses the negative summating potential recorded from the guinea pig cochlea. Hear Res 1990; 46: 277-87.

Bobbin RP, Konishi T. Acetylcholine mimics crossed olivocochlear bundle stimulation. Nat New Biol 1971; 231: 222-3.

Boettcher FA. Diltiazem does not protect the ear from noise-induced hearing loss in mongolian gerbils. Laryngoscope 1996; 106: 772-6.

Boettcher FA, Caldwell RK, Gratton MA, White DR, Miles LR. Effects of nimodipine on noise-induced hearing loss. Hear Res 1998; 121: 139-46.

Borg E, Canlon B, Engström B. Noise-induced hearing loss: Literature review and experiments in rabbits. Scandi Audiol (Suppl) 1995; 24: 1-147.

Bose R, Schnell CL, Pinsky C, Zitko V. Effects of excitotoxins on free radical indices in mouse brain. Toxicol Lett 1992; 60: 211-9.

Bullock R. Strategies for neuroprotection with glutamate antagonists. Ann NY Acad Sci 1995; 765: 272-78, 298.

Carpenter AM, Muchow D, Goycoolea MV. Ultrastructural studies of the human round window membrane. Arch Otolaryngol Head Neck Surg 1989; 115: 585-90.

Chen Z, Duan M, Lee H, Ruan R, Ulfendahl M. Pharmacokinetics of caroverine in the inner ear and its effects on cochlear function after systemic and local administrations in guinea pigs. Audiol Neuro-otol 2003; 8: 49-56.

Chen Z, Duan M, Ruan R, Ulfendahl M. Protection of auditory function against noise trauma with local caroverine administration in guinea pigs. Hear Res 2004; (in press).

Chen GD, Kong J, Reinhard K, Fechter LD. NMDA receptor blockage protects against permanent noise-induced hearing loss but not its potentiation by carbon monoxide. Hear Res 2001; 154: 108-15.

Choi DW. Ionic dependence of glutamate neurotoxicity. J Neurosci 1987; 7: 369-79.

Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1988; 1: 623-34.

Clark WA Jr. Average response computer (ARC-1). Quarterly progress report no. 49. Research laboratory of electronics. Cambridge, MA: MIT press. 1958.

Clark WA Jr, Goldstein MH, Brown MH, Molnar CE, O'Brien DF, Zieman HE. The average response computer (ARC): A digital device for computer averages and amplitudes and time histograms of electrophysiological responses. Transactions of IRE. 1961; 8: 46-51.

Clerici WJ, DiMartino DL, Prasad MR. Direct effects of reactive oxygen species on cochlear outer hair cell shape in vitro. Hear Res 1995; 84: 30-40.

Clerici WJ, Yang L. Direct effects of intraperilymphatic reactive oxygen species generation on cochlear function. Hear Res 1996; 101: 14-22.

Coles RR, Thompson AC, O'Donoghue GM. Intra-tympanic injections in the treatment of tinnitus. Clin Otolaryngol 1992; 17: 240-2.

Colletti V, Sittoni V, Shaddock LC. An experimental study of inner ear pathology due to NaCl on the round window membrane. Acta Otolaryngol (Stockh) 1986; 101: 53-8.

Cousillas H, Cole KS, Johnstone BM. Effect of spider venom on cochlear nerve activity consistent with glutamatergic transmission at hair cell-afferent dendrite synapse. Hear Res 1988; 36: 213-20.

Davis H, Davis PA, Loomis AL, Harvey EN, Hobart G. Electrical reactions of the human brain to auditory stimulation during sleep. J Neurophysiol 1939; 2: 500-14.

Davis H, Derbyshire AJ, Kemp EH, Lurie MH, Upton M. Experimental stimulation deafness. Science 1935; 81: 101-2.

Davis H, Morgan CT, Hawkins JE, Galambos R, Smith FW. Temporary deafness following exposure to loud tones and noise. Acta Otolaryngol (Suppl) 1950; 88: 4-57.

Delbarre G, Delbarre B, Calinon F, Ferger A. Accumulation of amino acids and hydroxyl free radicals in brain and retina of gerbil after transient ischemia. J Ocul Pharmacol 1991; 7: 147-55.

Denk DM, Heinzl H, Franz P, Ehrenberger K. Caroverine in tinnitus treatment: A placebo-controlled blind study. Acta Otolaryngol (Stockh) 1997; 117: 825-30.

Denk W, Holt JR, Shepherd GM, Corey DP. Calcium imaging of single stereocilia in hair cells: localization of transduction channels at both ends of tip links. Neuron 1995; 15: 1311-21.

Devau G, Lehouelleur J, Sans A. Glutamate receptors on type I vestibular hair cells of guinea-pig. Eur J Neurosci 1993; 5: 1210-7.

Doble A. The role of excitotoxicity in neurodegenerative disease: implications for therapy. Pharmacol Ther 1999; 81: 163-221.

Duan ML, Agerman K, Ernfors P, Canlon B. Complementary roles of neurotrophin 3 and an N-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. Proc Natl Acad Sci USA 2000; 97: 7597-602.

Duan ML, Canlon B. Forward masking is dependent on inner hair cell activity. Audiol Neurootol 1996; 1: 320-7.

Duan ML, Ulfendahl M, Laurell G, Counter AS, Pyykkö I, Borg E, Rosenhall U. Protection and treatment of sensorineural hearing disorders caused by exogenous

factors: Experimental findings and potential clinical application. Hear Res 2002; 169: 169-78.

Dulon D, Sugasawa M, Blanchet C, Erostegui C. Direct measurements of Ca (2+)-activated K+ currents in inner hair cells of the guinea-pig cochlea using photolabile Ca2+ chelators. Pflugers Arch 1995; 430: 365-73.

Dykens JA. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: implications for neurodegeneration. J Neurochem 1994; 63: 584-91.

Ehrenberger K. Clinical experience with caroverine in inner ear diseases. Adv Otorhinolaryngol 2002; 59: 156–62.

Ehrenberger K, Felix D. Glutamate receptors in afferent cochlear neurotransmission in guinea pigs. Hear Res 1991; 52: 73-80.

Ehrenberger K, Felix D. Caroverine depresses the activity of cochlear glutamate receptors in guinea pigs: In vivo model for drug-induced neuroprotection? Neuropharmacol 1992; 31: 1259-63.

Ehrenberger K, Felix D. Receptor pharmacological modes for inner ear therapies with emphasis on glutamate receptors: A survey. Acta Otolaryngol (Stockh) 1995; 115: 236-40.

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 1994; 79: 705-15.

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J. Alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular

and cochlear mechanosensory hair cells. Proc Natl Acad Sci U S A 2001; 98: 3501-6.

Evans PH. Free radicals in brain metabolism and pathology. Br Med Bull 1993; 49: 577-87.

Eybalin M, Altschuler RA. Immunoelectron microscopic localization of neurotransmitters in the cochlea. J Electron Microsc Tech 1990; 15: 209-24.

Eybalin M, Pujol R. A radioautographic study of [3H] L-glutamate and [3H] L-glutamine uptake in the guinea-pig cochlea. Neuroscience 1983; 9: 863-71.

Eybalin M, Pujol R. Choline acetyltransferase (ChAT) immunoelectron microscopy distinguishes at least three types of efferent synapses in the organ of Corti. Exp Brain Res 1987; 65: 261-70.

Eybalin M, Parnaud C, Geffard M, Pujol R. Immunoelectron microscopy identifies several types of GABA-containing efferent synapses in the guinea-pig organ of Corti. Neuroscience 1988; 24: 29-38.

Eybalin M, Pujol R. Cochlear neuroactive substances. Arch Otorhinolaryngol 1989: 246; 228-34.

Eybalin M. Neurotransmitters and neuromodulators in the mammalian cochlea. Physiol Rev 1993; 73: 309-73.

Felix D, Ehrenberger K. A microiontophoretic study of the role of excitatory amino acids at the afferent synapses of mammalian inner ear hair cells. Eur Arch Otorhinolaryngol 1990; 248: 1-3.

Felix D, Ehrenberger K. N-methyl-D-aspartate-induced oscillations in excitatory afferent neurotransmission in the guinea pig cochlea. Eur Arch Otorhinolaryngol 1991; 248: 429-31.

Felix D, Ehrenberger K. The efferent modulation of mammalian inner hair cell afferents. Hear Res 1992; 64: 1-5.

Flock Å. Electron microscopic and eletrophysiological studies on the lateral line canal organ. Acta Otolaryngol (Suppl) 1965; 199: 1-90.

Fredelius L. Time sequence of degeneration pattern of the organ of Corti after acoustic overstimulation. A transmission electron microscopy study. Acta Otolaryngol 1988; 106: 373-85.

Fredelius L, Rask-Andersen H, Johansson B, Urquiza R, Bagger-Sjoback D, Wersall J. Time sequence of degeneration pattern of the organ of Corti after acoustic overstimulation. A light microscopical and electrophysiological investigation in the guinea pig. Acta Otolaryngol 1988; 106: 81-93.

Furness DN, Lehre KP. Immunocytochemical localization of a high-affinity glutamate-aspartate transporter, GLAST, in the rat and guinea pig cochlea. Eur J Neurosci 1997; 9: 1961-9.

Gill R, Brazell C, Woodruff GN, Kemp JA. The neuroprotective action of dizocilpine (MK-801) in the rat middle cerebral artery occlusion model of focal ischaemia. Br J Pharmacol 1991; 103: 2030-6.

Glowatzki E, Fuchs PA. Transmitter release at the hair cell ribbon synapse. Nat Neurosci 2002; 5: 147-54.

Glowatzki E, Wild K, Brandle U, Fakler G, Fakler B, Zenner HP, Ruppersberg JP. Cell-specific expression of the alpha 9 n-ACh receptor subunit in auditory hair cells revealed by single-cell RT-PCR. Proc R Soc Lond B Biol Sci 1995; 262: 141-7.

Goycoolea MV, Lundman L. Round window membrane - Structure function and permeability: A review. Microsc Res Tech 1997; 36: 201-11.

Goycoolea MV, Muchow D, Schachern P. Experimental studies on round window structure: function and permeability. Laryngoscope 1988; 98 (6 Pt 2 Suppl 44): 1-20.

Goycoolea MV, Muchow DC, Schirber CM, Goycoolea HG, Schellhas K. Anatomical perspective, approach, and experience with multichannel intracochlear implantation. Laryngoscope 1990; 100 (2 Pt 2 Suppl 50): 1-18.

Goycoolea MV . Clinical aspects of round window membrane permeability under normal and pathological conditions. Acta Otolaryngol 2001; 121: 437-47.

Greenwood DD. The intensitive DL of tones: dependence of signal/masker ratio on tone level and on spectrum of added noise. Hear Res 1993; 65: 1-39.

Green GE, Khan KM, Beisel DW, Drescher MJ, Hatfield JS, Drescher DG. Calcium channel subunits in the mouse cochlea. J Neurochem 1996; 67: 37-45.

Guild SR. The circulation of the endolymph. Am J Anat 1977; 39: 57-81.

Gulley RL, Fex J, Wenthold RJ. Uptake of putative neurotransmitters in the organ of Corti. Acta Otolaryngol 1979; 88: 177-82.

Habermann J. Ueber die Schwerhörigkeit der Kesselschmiede. Arch f Ohreheilk Bd 1890; XXX: 1-25.

Halliwell B, Gutteridge JMC. Free radicals in Biology and Medicine, 2nd ed. 1989. Clarendon Press, Oxford.

Hamernik RP, Patterson JH, Turrentine GA, Ahroon WA. The quantitative relation between sensory cell loss and hearing thresholds. Hear Res 1989; 38: 199-211.

Hecox K, Jacobson JT. Auditory evoked potentials. 1984; In Northern, J.L. (Ed.), Hearing disorders. Boston: little, Brown.

Heinrich UR, Maurer J, Mann W. Ultrastructural evidence for protection of the outer hair cells of the inner ear during intense noise exposure by application of the organic calcium channel blocker diltiazem. ORL J Otorhinolaryngol Relat Spec 1999; 61: 321-7.

Hibi T, Suzuki T, Nakashima T. Perilymphatic concentration of gentamicin administration intratympanically in guinea pigs. Acta Otolaryngol (Stockh) 2001; 121: 336-41.

Hisashi K, Komune S, Nakagawa T, Kimitsuki T, Komiyama S. Regulation of inner ear fluid in the guinea pig cochlea after the application of saturated NaCl solution to the round window membrane. Eur Arch Otorhinolaryngol 1999; 256: S2-S5.

Hoffer ME, Allen K, Kopke RD, Weisskopf P, Gottshall K, Wester D. Transtympanic versus sustained-release administration of gentamicin: Kinetics, morphology, and function. Laryngoscope 2001; 111: 1343-57.

Hornykiewicz O, Hitzenberger G, Zellner H. Experimentell-pharmakologische und klinische Untersuchung über ein neues Spasmolytikum "Spadon" (Prüfbez. P. 201-1). Wien. Klin. Wochenschr 1963; 11: 189–97.

Housley GD, Ashmore JF. Direct measurement of the action of acetylcholine on isolated outer hair cells of the guinea pig cochlea. Proc R Soc Lond B Biol Sci 1991; 244: 161-7.

Hu BH, Guo W, Wang PY, Henderson D, Jiang SC. Intense noise-induced apoptosis in hair cells of guinea pig cochleae. Acta Otolaryngol 2000; 120: 19-24.

Hu BH, Henderson D, Nicotera TM. Involvement of apoptosis in progression of cochlear lesion following exposure to intense noise. Hear Res 2002; 166: 62-71.

Hudspeth AJ. Mechanoelectrical transduction by hair cells in the acousticolateralis sensory system. Annu Rev Neurosci 1983; 6: 187-215.

Ison JR, Payman GH, Palmer MJ, Walton JP. Nimodipine at a dose that slows ABR latencies does not protect the ear against noise. Hear Res 1997; 106: 179-83.

Issa NP, Hudspeth AJ. Clustering of Ca2+ channels and Ca 2+-activated K+ channels at fluorescently labeled presynaptic active zones of hair cells. Proc Natl Acad Sci U S A 1994; 91: 7578-82.

Janssen R. Glutamate neurotoxicity in the developing rat cochlea is antagonized by kynurenic acid and MK-801. Brain Res 1992; 590: 201-6.

Jäger W, Goiny M, Herrera-Marschitz M, Brundin L, Fransson A, Canlon B. Noise-induced aspartate and glutamate efflux in the guinea pig cochlea and hearing loss. Exp Brain Res 2000; 134: 426-34.

Jäger W, Goiny M, Herrera-Marschitz M, Flock Å, Hökfelt T, Brundin L. Sound-evoked efflux of excitatory amino acids in the guinea-pig cochlea in vitro. Exp Brain Res 1998; 121: 425-32.

Jacono AA, Hu B, Kopke RD, Henderson D, Van De Water TR, Steinman HM. Changes in cochlear antioxidant enzyme activity after sound conditioning and noise exposure in the chinchilla. Hear Res 1998; 117: 31-8.

Jewett DL, Romano MN, Williston JS. Human auditory evoked potentials: possible brain stem components detected on the scalp. Science 1970; 167: 1517-8.

Jewett DL, Williston JS. Auditory-evoked far fields averaged from the scalp of humans. Brain 1971; 94: 681-96.

Keithley EM, Ma CL, Ryan AF, Louis JC, Magal E. GDNF protects the cochlea against noise damage. Neuroreport 1998; 9: 2183-7.

Klinke R. Neurotransmission in the inner ear. Hear Res 1986; 22: 235-43.

Koch RA, Barish ME. Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. J Neurosci 1994; 14: 2585-93.

Kollmar R, Montgomery LG, Fak J, Henry LJ, Hudspeth AJ. Predominance of the alpha1D subunit in L-type voltage-gated Ca2+ channels of hair cells in the chicken's cochlea. Proc Natl Acad Sci U S A. 1997a; 94: 14883-8.

Kollmar R, Fak J, Montgomery LG, Hudspeth AJ. Hair cell-specific splicing of mRNA for the alpha1D subunit of voltage-gated Ca2+ channels in the chicken's cochlea. Proc Natl Acad Sci U S A 1997; 94: 14889-93.

Kopke R, Allen KA, Henderson D, Hoffer M, Frenz D, Van de Water T. A radical demise. Toxins and trauma share common pathways in hair cell death. Ann N Y Acad Sci 1999; 884: 171-91.

Kopke R, Staecker H, Lefebvre P, Malgrange B, Moonen G, Ruben RJ, Van de Water TR. Effect of neurotrophic factors on the inner ear: clinical implications. Acta Otolaryngol 1996; 116: 248-52.

Koppi S, Eberhardt G, Haller R, König P. Calcium-channel-blocking agent in the treatment of acute alcohol-withdrawal - Caroverine verus meprobamate in a randomized double blind study. Neuropsychobiol 1987; 17: 49-52.

Kujawa SG, Glattke TJ, Fallon M, Bobbin RP. Intracochlear application of acetylcholine alters sound-induced mechanical events within the cochlear partition. Hear Res 1992; 61: 106-16.

Lamm K, Arnold W. The effect of blood flow promoting drugs on cochlear blood flow, perilymphatic pO(2) and auditory function in the normal and noise-damaged hypoxic and ischemic guinea pig inner ear. Hear Res 2000; 141: 199-219.

Lawrence M, Wolsk D, Litton WB. Circulation of inner ear fluids. Ann Otol Rhinol Laryngol 1961; 70: 753-76.

Lefebvre PP, Malgrange B, Lallemend F, Staecker H, Moonen G, Van De Water TR. Mechanisms of cell death in the injured auditory system: otoprotective strategies. Audiol Neurootol 2002; 7: 165-70.

Lehner R, Brugger H, Maassen MM, Zenner HP. A totally implantable drug delivery system for local therapy of the middle and inner ear. Ear Nose Throat J 1997; 76: 567-70.

Li H, Buchan AM. Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. J Cereb Blood Flow Metab 1993; 13: 933-9.

Li HS, Niedzielski AS, Beisel KW, Hiel H, Wenthold RJ, Morley BJ. Identification of a glutamate/aspartate transporter in the rat cochlea. Hear Res 1994; 78: 235-42.

Lim DJ. Cochlear anatomy related to cochlear micromechanics. A review. J Acoust Soc Am 1980; 67: 1686-95.

Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 1994; 330: 613-22.

Liu Y, Fechter LD. MK-801 protects against carbon monoxide-induced hearing loss. Toxicol Appl Pharmacol 1995; 132: 196-202.

Lundquist PG. Aspects on endolymphatic sac morphology and function. Arch Otorhinolaryngol 1976; 212: 231-40.

Mann W, Pilgramm M, Lohle E, Beck C. Calcium antagonists and damage to the organ of Corti in acoustic trauma. HNO 1987; 35: 203-7.

Manzo RP, Gomez DG, Potts G. Cerebrospinal fluid absorption in the rabbit: inner ear pathway. Acta Otolaryngol (Stockh) 1990; 109: 389-96.

Martinez-Dunst C, Michaels RL, Fuchs PA. Release sites and calcium channels in hair cells of the chick's cochlea. J Neurosci 1997; 17: 9133-44.

Matsubara A, Laake JH, Davanger S, Usami S, Ottersen OP. Organization of AMPA receptor subunits at a glutamate synapse: A quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. J Neurosci 1996; 16: 4457-67.

Maurer J, Heinrich UR, Hinni M, Mann W. Alteration of the calcium content in inner hair cells of the cochlea of the guinea pig after acute noise trauma with and

without application of the organic calcium channel blocker diltiazem. ORL J Otorhinolaryngol Relat Spec 1999; 61: 328-33.

Maurer J, Riechelmann H, Amedee RG, Mann WJ. Diltiazem for prevention of acoustical trauma during otologic surgery. ORL J Otorhinolaryngol Relat Spec 1995; 57: 319-24.

McCabe BF. Autoimmune sensorineural hearing loss. Ann Otol Rhinol Laryngol 1979; 88: 585-9.

May JJ. Occupational hearing loss. Am J Ind Med 2000; 37: 112-20.

McCleskey EW. Calcium channels: cellular roles and molecular mechanisms. Curr Opin Neurobiol 1994; 4: 304-12.

McIntosh TK. Neurochemical sequelae of traumatic brain injury: therapeutic implications. Cerebrovasc Brain Metab Rev 1994; 6: 109-62.

Medina JE, Drescher DG. The amino-acid content of perilymph and cerebrospinal fluid from guinea pig and the effect of noise on the amino-acid composition of perilymph. Neuroscience 1981; 6: 505-09.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K, Mishina M. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 1992; 357: 70-4.

Meldrum B. Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission. Cerebrovasc Brain Metab Rev 1990; 2: 27-57.

Miriszlai E, Benedeczky I, Csapo S, Bodanszky H. The ultrastructure of the round window membrane of the cat. ORL J Otorhinolaryngol Relat Spec 1978; 40: 111-9.

Molinari GA. Alteration of inner ear mechanisms resulting from application of

sodium chloride to the round window membrane. Ann Otol Rhinol Laryngol 1972; 81: 315-22.

Moskowitz D, Lee KJ, Smith HW. Steroid use in idiopathic sudden sensorineural hearing loss. Laryngoscope 1984; 94: 664-6.

Muir KW, Lees KR. Clinical experience with excitatory amino acid antagonist drugs. Stroke 1995; 26: 503-513.

Naftalin L, Harrison MS. Circulation of labyrinthine fluids. J Laryngot Otol 1958; 72: 118-36.

Nakagawa T, Komune S, Uemura T, Akaike N. Excitatory amino acid response in isolated spiral ganglion cells of guinea pig cochlea. J Neurophysiol 1991; 65: 715-23.

Nicotera TM, Hu BH, Henderson D. The caspase pathway in noise-induced apoptosis of the chinchilla cochlea. J Assoc Res Otolaryngol 2003; 4: 466-77.

Niedzielski AS, Wenthold RJ. Expression of AMPA, kainate, and NMDA receptor subunits in cochlear and vestibular ganglia. J Neurosci 1995; 15: 2338-53.

Nordang L, Cestreicher E, Arnold W, Anniko M. Glutamate is the afferent neurotransmitter in the human cochlea. Acta Otolaryngol 2000; 120: 359-62.

Norris CH, Guth PS. The release of acetylcholine (ACH) by the crossed olivo-cochlear bundle (COCB). Acta Otolaryngol 1974; 77: 318-26.

Oestreicher E, Ehrenberger K, Felix D. Different action of memantine and caroverine on glutamatergic transmission in the mammalian cochlea. Adv Otorhinolaryngol 2002; 59: 18-25.

Ohinata Y, Yamasoba T, Schacht J, Miller JM. Glutathione limits noise-induced hearing loss. Hear Res 2000; 146: 28-34.

Ohlemiller KK, Wright JS, Dugan LL. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol 1999; 4: 229-36.

Olney JW. Neurotoxicity of excitatory amino acids. In: Kainic Acid as a Tool in Neurobiology, 1978; pp.95-112, McGeer EG, Onley JW, PL (eds.) Raven Press, New York.

Parnes LS, Sun AH, Freeman DJ. Corticosteroid pharmacokinetics in the inner ear fluids: An animal study followed by clinical application. Laryngoscope 1999; 109: 1-17.

Pellegrini-Giampietro DE, Cherici G, Alesiani M, Carla V, Moroni F. Excitatory amino acid release and free radical formation may cooperate in the genesis of ischemia-induced neuronal damage. J Neurosci 1990; 10: 1035-41.

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. Proc Natl Acad Sci USA 1992; 89: 10499-503.

Petralia RS, Wenthold RJ. Types of excitatory amino acid receptors and their localization in the nervous system and hypothalamus. In: Excitatory Amino Acids: Their Role in Neuroendocrine Function. Eds. Bann DW and Mahesh V.B., Boca Raton. CRC Press, 1995; pp 55-101.

Plontke SK, Wood AW, Salt AN. Analysis of gentamicin kinetics in fluids of the inner ear with round window administration. Otolo & Neurol 2002; 23: 967-74.

Prehn JH, Lippert K, Krieglstein J. Are NMDA or AMPA/kainate receptor antagonists more efficacious in the delayed treatment of excitotoxic neuronal injury? Eur J Pharmacol 1995; 292: 179-89.

Prigioni I, Masetto S, Russo G, Taglietti V. Calcium currents in solitary hair cells isolated from frog crista ampullaris.J Vestib Res 1992; 2: 31-9.

Pringle AK, Iannotti F, Wilde GJ, Chad JE, Seeley PJ, Sundstrom LE. Neuroprotection by both NMDA and non-NMDA receptor antagonists in in vitro ischemia. Brain Res 1997; 755: 36-46.

Puel JL. Chemical synaptic transmission in the cochlea. Prog Neurobiol 1995; 47: 449-76.

Puel JL, d'Aldin C, Ruel J, Ladrech S, Pujol R. Synaptic repair mechanisms responsible for functional recovery in various cochlear pathologies. Acta Otolaryngol (Stockh) 1997; 117: 214-8.

Puel JL, d'Aldin C, Safieddine S, Eybalin M, Pujol R. Excitotoxicity and plasticity of IHC-auditory nerve contributes to both temporary and permanent threshold shift. *In* Scientific Basis of Noise-Induced Hearing Loss, Axelsson A, Borchgrevink H, Hamernik RP, Hellstrom PA, Henderson D, Salvi RJ, Eds. 1996: 36-42. Thieme. New York.

Puel JL, Ladrech S, Chabert R, Pujol R, Eybalin M.Electrophysiological evidence for the presence of NMDA receptors in the guinea pig cochlea. Hear Res 1991; 51: 255-64.

Puel JL, Pujol R, Tribillac F, Ladrech S, Eybalin M. Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. J Comp Neurol 1994; 341: 241-56.

Puel JL, Ruel J, Gervais d'Aldin C, Pujol R. Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. Neuroreport 1998; 9: 2109-14.

Puel JL, Saffiedine S, Gervais d'Aldin C, Eybalin M, Pujol R. Synaptic regeneration and functional recovery after excitotoxic injury in the guinea pig cochlea. C R Acad Sci III 1995; 318: 67-75.

Pujol R, Lenoir M, Robertson D, Eybalin M, Johnstone BM. Kainic acid selectively alters auditory dendrites connected with cochlear inner hair cells. Hear Res 1985; 18: 145-51.

Pujol R, Puel JL, D'Aldin CG, Eybalin M. Pathophysiology of the glutamatergic synapses in the cochlea. Acta Otolaryngol (Stockh) 1993; 113: 330-4.

Pujol R, Puel JL, Eybalin M. Implication of non-NMDA and NMDA receptors in cochlear ischemia. Neuroreport 1992; 3: 299-302.

Pujol R, Rebillard G, Puel JL, Lenoir M, Eybalin M, Recasens M. Glutamate neurotoxicity in the cochlea: a possible consequence of ischaemic or anoxic conditions occurring in ageing. Acta Otolaryngol Suppl 1990; 476: 32-6.

Quirk WS, Seidman MD. Cochlear vascular changes in response to loud noise. Am J Otol 1995; 16: 322-5.

Quirk WS, Shivapuja BG, Schwimmer CL, Seidman MD. Lipid peroxidation inhibitor attenuates noise-induced temporary threshold shifts. Hear Res 1994; 74: 217-20.

Rebillard G, Ruel J, Nouvian R, Saleh H, Pujol R, Dehnes Y, Raymond J, Puel JL, Devau G. Glutamate transporters in the guinea-pig cochlea: partial mRNA sequences, cellular expression and functional implications. Eur J Neurosci 2003; 17: 83-92.

Richardson TL, Ishiyama E, Keels EW. Submicroscopic studies of the round window membrane. Acta Otolaryngol 1971; 71: 9-21.

Robertson D. Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hear Res 1983; 9: 263-78.

Robertson D, Johnstone BM. Effect of divalent cations on spontaneous and evoked activity of single mammalian auditory neurones. Pflugers Arch 1979; 380: 7-12.

Robles L, Ruggero MA. Mechanics of the mammalian cochlea. Physiol Rev 2001; 81: 1305-52.

Ruel J, Bobbin RP, Vidal D, Pujol R, Puel JL. The selective AMPA receptor antagonist GYKI 53784 blocks action potential generation and excitotoxicity in the guinea pig cochlea. Neuropharmacol 2000; 39: 1959-73.

Ruel J, Chen C, Pujol R, Bobbin RP, Puel JL. AMPA-preferring glutamate receptors in cochlear physiology of adult guinea pig. J Physiol 1999; 518 (Pt 3): 667-80.

Ruel J, Nouvian R, Gervais d'Aldin C, Pujol R, Eybalin M, Puel JL. Dopamine inhibition of auditory nerve activity in the adult mammalian cochlea. Eur J Neurosci 2001; 14: 977-86.

Ryan AF, Brumm D, Kraft M. Occurrence and distribution of non-NMDA glutamate receptor mRNAs in the cochlea. Neuroreport 1991; 2: 643-6.

Ryan A, Dallos P. Effect of absence of cochlear outer hair cells on behavioural auditory threshold. Nature 1975; 253: 44-6.

Safieddine S, Eybalin M. Co-expression of NMDA and AMPA/kainate receptor mRNAs in cochlear neurones. Neuroreport 1992; 3: 1145-8.

Saletu B, Grunberger J, Anderer P, Linzmayer L, König P. Acute central effects of the calcium channel blocker and antiglutamatergic drug caroverine. Arzneimittelforschung/Drug Res 1995; 45: 217-29.

Saletu B, Grunberger J, Anderer P, Linzmayer L, König P. On the cerebro-protective effects of caroverine, a calcium-channel blocker and antiglutamatergic drug: double-blind, placebo-controlled, EEG mapping and psychometric studies under hypoxia. Br J Clin Pharmacol 1996; 41: 89-99.

Salt AN, Ma Y. Quantification of solute entry into cochlear perilymph through the round window membrane. Hear Res 2001; 154: 88-97.

Salvi RJ, Shulman A, Stracher A, Ding D, Wang J. Protecting the inner ear from acoustic trauma. Int Tinnitus J 1998; 4: 11-15.

Saunders JC, Rhyne RL. Cochlear nucleus activity and threshold shift in cat. Brain Res 1970; 24: 336-39.

Saunders JC, Dear SP, Schneider ME. The anatomical consequences of acoustic injury: A review and tutorial. J Acoust Soc Am 1985; 78: 833-60.

Scarpa A. Anatomical observations on the round window. Arch Otolaryngol Head Neck Surg 1962; 75: 24-59.

Schacht J, Zenner HP. Evidence that phosphoinositides mediate motility in cochlear outer hair cells. Hear Res 1987; 31: 155-9.

Scheibe F, Haupt H. Biochemical differences between perilymph, cerebrospinal fluid and blood plasma in the guinea pig. Hear Res 1985; 17: 61-6.

Schoendorf J, Neugebauer P, Michel O. Continuous intratympanic infusion of gentamicin via a microcatheter in Ménière's disease. Otolaryngol Head Neck Surg 2001; 124: 203-07.

Schuknecht H, Churchill J, Dorean R. The localization of acetylcholinesterase in the cochlea. Arch Otolaryngol (Stockh) 1959; 69: 549-59.

Seidman MD. Effects of dietary restriction and antioxidants on presbyacusis. Laryngoscope. 2000; 110: 727-38.

Seidman MD. Glutamate antagonists, steroids, and antioxidants as therapeutic options for hearing loss and tinnitus and the use of an inner ear drug delivery system. Int Tinnitus J 1998; 4: 148-54.

Seidman MD, Khan MJ, Bai U, Shirwany N, Quirk WS. Biologic activity of mitochondrial metabolites on aging and age-related hearing loss. Am J Otol 2000; 21: 161-7.

Seidman MD, Shivapuja BG, Quirk WS. The protective effects of allopurinol and superoxide dismutase on noise-induced cochlear damage. Otolaryngol Head Neck Surg 1993; 109: 1052-6.

Sheardown MJ, Nielsen EO, Hansen AJ, Jacobsen P, Honore T. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxaline: a neuroprotectant for cerebral ischemia. Science 1990; 247: 571-4.

Shinomori Y, Spack DS, Jones DD, Kimura RS. Volumetric and dimensional analysis of the guinea pig inner ear. Ann Otol Rhinol Laryngol 2001; 110: 91-98.

Shirwany NA, Seidman MD, Tang W. Effect of transtympanic injection of steroids on cochlear blood flow, auditory sensitivity, and histology in the guinea pig. Am J Otol 1998; 19: 230-5.

Shoji F, Miller AL, Mitchell A, Yamasoba T, Altschuler RA, Miller JM. Differential protective effects of neurotrophins in the attenuation of noise-induced air cell loss. Hear Res 2000a; 146: 134-42.

Shoji F, Yamasoba T, Magal E, Dolan DF, Altschuler RA, Miller JM. Glial cell line-derived neurotrophic factor has a dose dependent influence on noise-induced hearing loss in the guinea pig cochlea. Hear Res 2000b; 142: 41-55.

Shulman A. Noise, calpain, calpain inhibitors, and neuroprotection: a preliminary report of tinnitus control. Int Tinnitus J 1998; 4: 134-40.

Silverstein H, Arruda J, Rosenberg SI, Deems D, Hester TO. Direct round window membrane application of gentamicin in the treatment of Ménière's disease. Otolaryngol Head Neck Surg 1999; 120: 649-55.

Silverstein H, Choo D, Rosenberg S, Kuhn J, Seidman M, Stein I. Intratympanic steroid treatment of inner ear disease and tinnitus. Ear Nose Throat J 1996; 75: 468-88.

Simonian NA, Coyle JT. Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 1996; 36: 83-106.

Solberg Y, Rosner M, Turetz J, Belkin M. MK-801 has neuroprotective and antiproliferative effects in retinal laser injury. Invest Ophthalmol Vis Sci 1997; 38: 1380-9.

Spandow O, Hellstrom S, Anniko M. Impaired hearing following instillation of hydrocortisone into the middle ear. Preliminary report from an animal study. Acta Otolaryngol Suppl 1988; 455: 90-3.

Spedding M, Paoletti R. Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacol Rev 1992; 44: 363-76.

Spoendlin H. Primary structural changes in the organ of corti after acoustic overstimulation. Acta Otolaryngol (Stockh) 1971; 71: 166-76.

Spoendlin H. Innervation densities of the cochlea. Acta Otolaryngol 1972; 73: 235-48.

Stebbins WC, Hawkins JE Jr, Johnson LG, Moody DB. Hearing thresholds with outer and inner hair cell loss. Am J Otolaryngol 1979; 1: 15-27.

Sterkers O, Ferrary E, Amiel C. Production of inner ear fluids. Physiol Rev 1988; 68: 1083-128.

Stover T, Yagi M, Raphael Y. Cochlear gene transfer: round window versus cochleostomy inoculation. Hear Res 1999; 136: 124-30.

Stracher A. Calpain inhibitors as therapeutic agents in nerve and muscle degeneration. Ann N Y Acad Sci 1999; 884: 52-9.

Su ZL, Jiang SC, Gu R, Yang WP. Two types of calcium channels in bullfrog saccular hair cells. Hear Res 1995; 87: 62-8.

Taylor W, Pearson J, Mair A, Burns W. Study on noise and hearing in jute weaving. J Acoust Soc Am 1965; 32: 135-7.

Thalmann I, Comegys TH, Liu SZ, Ito Z, Thalmann R. Protein profiles of perilymph and endolymph of the guinea pig. Hear Res 1992; 63: 37-42.

Toynbee J. The disease of the ear. J.C. Churchill, London. 1860.

Tucker T, Fettiplace R. Confocal imaging of calcium microdomains and calcium extrusion in turtle hair cells. Neuron 1995; 15: 1323-35.

Udilova N, Kozlov AV, Bieberschulte W, Frei K, Ehrenberger K, Nohl H. The antioxidant activity of caroverine. Biochem Pharmacol 2003; 65: 59-65.

Ulehlova L, Voldrich L, Janisch R. Correlative study of sensory cell density and cochlear length in humans. Hear Res 1987; 28: 149-51.

Ulfendahl M. Motility in auditory sensory cells. Acta Physiol Scand. 1987; 130: 521-7.

Usami S, Matsubara A, Fujita S, Shinkawa H, Hayashi M. NMDA (NMDAR1) and AMPA-type (GluR2/3) receptor subunits are expressed in the inner ear. NeuroReport 1995; 6: 1161-4.

Vetter DE, Liberman MC, Mann J, Barhanin J, Boulter J, Brown MC, Saffiote-Kolman J, Heinemann SF, Elgoyhen AB. Role of alpha9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. Neuron 1999; 23: 93-103.

Von Békésy G. Experiments in hearing. New York: McGraw-Hill, 1960.

Warr WB, Guinan JJ Jr. Efferent innervation of the organ of corti: two separate systems. Brain Res 1979; 173: 152-5.

Wang J, Dib M, Lenoir M, Vago P, Eybalin M, Hameg A, Pujol R, Puel JL. Riluzole rescues cochlear sensory cells from acoustic trauma in the guinea pig. Neuroscience 2002; 111: 635-48.

Wang J, Ding D, Shulman A, Stracher A, Salvi RJ. Leupeptin protects sensory hair cells from acoustic trauma. Neuroreport 1999; 10: 811-6.

Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel JL, Zine A. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. J Neurosci 2003; 23: 8596-607.

Weisstaub N, Vetter DE, Elgoyhen AB, Katz E. The alpha9alpha10 nicotinic acetylcholine receptor is permeable to and is modulated by divalent cations. Hear Res 2002; 167: 122-35.

Yagi M, Magal E, Sheng Z, Ang KA, Raphael Y. Hair cell protection from aminoglycoside ototoxicity by adenovirus-mediated overexpression of glial cell line-derived neurotrophic factor. Hum Gene Ther 1999; 10: 813-23.

Yamane H, Nakai Y, Takayama M, Iguchi H, Nakagawa T, Kojima A. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur Arch Otorhinolaryngol 1995; 252: 504-8.

Yamasoba T, Harris C, Shoji F, Lee RJ, Nuttall AL, Miller JM. Influence of intense sound exposure on glutathione synthesis in the cochlea. Brain Res 1998; 804: 72-8.

Yamasoba T, Nuttall AL, Harris C, Raphael Y, Miller JM. Role of glutathione in protection against noise-induced hearing loss. Brain Res 1998; 784: 82-90.

Yamasoba T, Schacht J, Shoji F, Miller JM. Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res 1999; 815: 317-25.

Zenner HP, Zimmermann U, Schmitt U. Reversible contraction of isolated mammalian cochlear hair cells. Hear Res 1985; 18: 127-33.

Zhang SY, Robertson D, Yates G, Everett A. Role of L-type Ca2+ channels in transmitter release from mammalian inner hair cells I. Gross sound-evoked potentials. J Neurophysiol. 1999; 82: 3307-15.

Zidanic M, Fuchs PA. Kinetic analysis of barium currents in chick cochlear hair cells. Biophys J 1995; 68: 1323-36.

Zivin JA, Choi DW. Stroke therapy. Sci Am 1991; 265: 56-63.

Original Paper

Audiology & Neuro-Otology

Audiol Neurootol 2003;8:49–56
DOI: 10.1159/000067893

Received: March 6, 2002
Accepted after revision: July 25, 2002

Pharmacokinetics of Caroverine in the Inner Ear and Its Effects on Cochlear Function after Systemic and Local Administrations in Guinea Pigs

Zhiqiang Chen[a,c] Maoli Duan[c] Howsung Lee[b] Runsheng Ruan[a]
Mats Ulfendahl[c]

Departments of [a]Otolaryngology and [b]Pharmacology, National University of Singapore, Singapore;
[c]Department of Clinical Neuroscience and Center for Hearing and Communication Research, Karolinska Institutet, Stockholm, Sweden

Key Words
Caroverine · Glutamate · Pharmacokinetics · Round window membrane · Perilymph · Cerebrospinal fluid · Plasma · High-performance liquid chromatography · Auditory brainstem response · Guinea pig

Abstract
Caroverine, an N-methyl-*D*-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist, has been shown to protect the inner ear from excitotoxicity and to be effective in the treatment of cochlear synaptic tinnitus. Local administration of caroverine on the round window membrane (RWM) could be a more effective means of administration to avoid systemic side/adverse effects. The present study investigates the pharmacokinetics of caroverine in the perilymph, cerebrospinal fluid (CSF) and plasma following intravenous and local applications at different dosages. High-performance liquid chromatography was used to determine the drug concentrations. Our results show much higher caroverine concentrations in the perilymph with lower concentrations in CSF and plasma following local applications compared with systemic administration. Auditory brainstem responses were measured to evaluate the changes in auditory function. The effects on hearing were transient and fully reversible 24 h after local caroverine applications. The findings suggest that local application of caroverine on the RWM for the treatment of excitotoxicity-related inner ear diseases, such as tinnitus and noise-induced hearing loss, might be both safe and more efficacious while avoiding high blood and CSF caroverine levels seen with systemic administration.

Introduction

Glutamate has been shown to be the most likely neurotransmitter at the synapses between the inner hair cells and afferent neurons in the mammalian cochlea [Altschuler et al., 1989; Eybalin and Pujol, 1989; Felix and Ehrenberger, 1990; Eybalin, 1993; Puel, 1995; Ruel et al., 1999; Glowatzki and Fuchs, 2002]. Using immunocytochemistry and in situ hybridization, 3 types of postsynaptic glutamate receptors have been identified: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-*D*-aspartate (NMDA) [Ryan et al., 1991; Usami et al., 1995; Matsubara et al., 1996; Niedzielski and Wenthold, 1995]. Under pathological condi-

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1420–3030/03/0081–0049$19.50/0

Accessible online at:
www.karger.com/aud

Maoli Duan, MD, PhD
Center for Hearing and Communication Research
Building M1, Karolinska Hospital
SE–171 76 Stockholm (Sweden)
Tel. +46 8 51773210, Fax +46 8 348546, E-Mail maoli.duan@cfh.ki.se

tions, such as noise trauma and ischemia, excessive glutamate is released and causes cellular damage through the process described as excitotoxicity in the mammalian cochlea [Pujol et al., 1993]. A variety of inner ear disorders, such as noise-induced hearing losses, sudden hearing loss, presbycusis and cochlear synaptic tinnitus, could be linked to the excitotoxic effect of glutamate [Puel, 1995]. Glutamate receptor antagonists have been shown to protect the inner ear from excitotoxicity [Pujol et al., 1993; Duan et al., 2000, 2002] and thus may be a promising therapeutic modality for a variety of inner ear diseases [Ehrenberger and Felix, 1995]. Unfortunately, most of the glutamate receptor antagonists have intolerable neuropsychiatric adverse effects and cannot be administered systemically [Lipton and Rosenberg, 1994; Bullock, 1995; Muir and Lees, 1995].

Caroverine (Spasmium®, Phafag AG), a quinoxaline derivative, is clinically available in some countries as a spasmolytic drug. Microiontophoretic experiments in guinea pigs have demonstrated that caroverine acts as a specific, but reversible antagonist of NMDA and AMPA receptors in the cochlear afferents [Ehrenberger and Felix, 1992]. Caroverine is a promising drug as a glutamate receptor antagonist. In a study on 60 patients suffering from tinnitus with a probable cochlear origin, 63.3% of the patients responded positively to a single infusion of caroverine. The beneficial effect of the caroverine therapy was still present after 1 week in 43.3% of the patients [Denk et al., 1997]. Although its safety has been documented [Koppi et al., 1987; Saletu et al., 1995], the potential adverse effects of glutamate receptor antagonists should be considered when applied systemically. In addition, the therapeutic effect of systemic caroverine may not be ideal at nontoxic doses because of its limited ability to penetrate the blood-labyrinth barrier. Thus, local application of caroverine onto the round window membrane (RWM) might be an alternative since the drug may enter the inner ear directly, resulting in higher perilymph concentrations and reduced systemic absorption and toxicity. For clinical application, basic information about the rate of drug diffusion across the RWM, systemic caroverine absorption and elimination of drug from the inner ear is necessary. The present study was designed to investigate the pharmacokinetics of caroverine in the perilymph, cerebrospinal fluid (CSF) and plasma after systemic and local applications at different dosages. Auditory brainstem response (ABR) measurements were performed to evaluate the effects on auditory function after local applications.

Materials and Methods

Albino guinea pigs of either sex (300–400 g) were used. The care for and use of the animals in this study were approved by the Ethical Committees at the National University of Singapore and the Karolinska Institutet in Stockholm. The animals were anesthetized by intramuscular injection with a mixture of ketamine (40 mg/kg) and xylazine (4 mg/kg). For a pharmacokinetic study, the animals were randomly assigned to 3 groups: 1 group for intravenous injection (IV) and 2 groups for local applications onto the RWM with a low dose (LD) and the other a high dose (HD). Intravenous injection was administered through the femoral vein with the dose of 4 mg/kg body weight at the concentration of 1.6 mg/ml in normal saline. For local applications, two doses were used: 15 µl of 1.6 mg/ml (LD) and 12.8 mg/ml (HD) of caroverine in normal saline. Under an operating microscope, the right temporal bulla was opened through a postauricular incision to expose the round window, and a small piece of gelfoam was placed on the RWM. Fifteen microliters of caroverine at the concentrations of either 1.6 or 12.8 mg/ml were dropped onto the gelfoam. The hole of the temporal bulla was then closed using dental cement (Fuji I, Japan). CSF, plasma and perilymph were sampled at 10, 30, 60, 180 and 360 min after administration. Three animals were used at each time point (total of 45 animals).

CSF, Plasma and Perilymph Sampling

CSF Sampling. The animals were anesthetized with ketamine and xylazine, and in addition, local anesthesia with xylocaine was given. An incision was made through the skin and muscle of the dorsal neck. Twenty microliters of CSF were collected from the subarachnoid space at the foramen magnum with a 1-ml syringe connected to a 29-gauge needle.

Plasma Sampling. After CSF sampling, a 3-ml blood sample was collected by heart puncture through the thoracic cavity using a 5-ml syringe. The plasma was obtained by centrifugation of the blood sample at 3,000 rpm for 5 min.

Perilymph Sampling. In order to avoid CSF contamination, the animals were deeply anesthetized with ketamine and xylazine, the perilymph was then collected after decapitation. The bulla was removed from the skull base and opened to expose the middle ear. The remaining gelfoam was removed, and the RWM and middle ear cavity were rinsed 4 times with 30% methanol within 2 min. To make sure there was no contamination of perilymph by the residual caroverine in the middle ear cavity, the last wash was collected for detection of caroverine using high-performance liquid chromatography (HPLC), and only perilymph samples with no detectable caroverine in the last wash were used for analysis. Six to 10 µl of perilymph were collected through the RWM with a glass capillary after complete removal of the solution in the middle ear cavity. In the ears of the animals given the drug systemically, the samples were obtained in the same way without local RWM rinsing. All of the samples were stored at –20°C for HPLC analysis within 1 week.

HPLC Analysis

The concentration analysis was performed on a Hewlett Packard (HP) 1050 HPLC system equipped with chemstation and HP 1100 UV detector set at 230 nm. A guard column (4.6 × 12.5 mm, 5 µm, HP) was connected to the HPLC column (Hypersil BDS C18, 5 µm, 150 × 4.6 mm). Drug concentrations with samples were determined from calibration curves obtained by plotting the chromatographic peak area ratio of caroverine/internal standardization described

below. Peak areas were computed from the HP HPLC system software chemstation.

The mobile phase for caroverine in the perilymph and CSF was 34% of acetonitrile and 66% of the mixture of 0.02 mol KH_2PHO_4 and 1.5 ml of diethylamine in 1 liter of deionized water adjusted to pH 5.7 with 1 *N* HCl. For caroverine in plasma, the mobile phase was 30% of acetonitrile and 70% of the mixture of 0.02 mol KH_2PHO_4 and 1.5 ml of diethylamine in 1 liter of deionized water adjusted to pH 5.9 with 1 *N* HCl. The flow rate was 1 ml/min. Standard stock solutions of caroverine-hydrochloride and flunitrazepam (internal standard; both 1 mg/ml) were prepared in 100% methanol and stored at 4°C for not more than 2 weeks. Calibration samples were prepared at different concentrations by diluting the stock solution with normal saline. At least 5 caroverine calibrators in 20 µl saline, 20 µl of control CSF samples or 10 µl of control perilymph samples (diluted to 20 µl with normal saline) were mixed with 10 µl of flunitrazepam of appropriate concentrations. The mixture was vortexed and directly injected into the HPLC system. Different calibration curves had to be used because of the wide range of concentrations in the perilymph (calibration range: from 10 ng/ml to 9.6 µg/ml). Linear calibration curves were obtained. For caroverine in plasma, calibration samples were prepared in 100 µl of control plasma samples. Two hundred microliters of acetonitrile and 20 µl of flunitrazepam of appropriate concentrations were added to 20 µl of caroverine hydrochloride calibrators (calibration range: 10–960 ng/ml) and 100 µl of control plasma samples. The mixture was centrifuged to precipitate plasma proteins. After centrifugation, the supernatant was evaporated with a stream of nitrogen air and the residue was reconstituted in 40 µl of mobile phase for injection. The retention times of caroverine and flunitrazepam were 13.1 and 18.0 min, respectively, in plasma, and 8.6 and 11.9 min, respectively, in the perilymph and CSF. The inter-day coefficient of variation ranged from 4.6 to 16.1%.

Using the above conditions, the limit of quantification was 10 ng/ml. This HPLC method for analysis of caroverine in the perilymph, CSF and plasma is reproducible and sensitive. No interference from endogenous substances or the anesthetic agents was encountered.

ABR Measurements

For the animals with local applications of caroverine, ABR measurements were performed before and at 30 min, 3 h, 6 h and 24 h after the application. Three groups were studied: LD and HD groups and a control group. Each group included 5 animals. In the control group, 15 µl of normal saline was applied with the gelfoam onto the RWM. In the LD and HD local groups, the caroverine administrations were the same as in the pharmacokinetic study. The animals were anesthetized as above. ABR measurements were performed in a sound-proof booth as described previously [Duan et al., 2000]. Responses were recorded with subcutaneous stainless electrodes as the potential difference between an electrode on the vertex and an electrode on the mastoid, while the leg served as ground. The body temperature of the animals was maintained at 38°C by using an isothermic heating pad. Stimulus intensity was calibrated with a ¼-inch condenser microphone (Bruël & Kjær Instruments, Marlborough, Mass., USA, model 4135) and all of the sound pressure levels were expressed in decibels relative to 20 µPa. The stimulus signal was generated through Tucker-Davis Technologies (Gainesville, Fla., USA) equipment controlled by a computer and delivered by an earphone (Telephonics TDH 39, Farmingdale, N.Y., USA). The stimuli were delivered through a closed acoustic system sealed into the external auditory meatus. The evoked response was amplified 100,000 times and averaged 2048 sweeps in real time by a digital signal processor (DSP32C) with a time domain artifact rejection. The initial intensity of the stimulus was 90 dB peak sound pressure level and was then decreased in 10-dB steps until the threshold was approached and then in 5-dB steps until the ABR disappeared. Threshold was defined as the lowest intensity at which a visible ABR wave III was seen in two averaged runs since the wave III was the largest wave in guinea pigs. Threshold was measured at 4 frequencies: 20, 16, 12.5 and 8 kHz. The latency and amplitude of wave I at 90 dB of each frequency were recorded. The differences in mean values of thresholds, latencies and amplitudes between pre- and posttreatment measurements and between different groups were tested for significance ($p < 0.05$) by Student's two-tailed t test.

Results

Pharmacokinetics of Caroverine

Local administrations resulted in dramatically higher levels of perilymph caroverine concentration than that seen after intravenous injection (fig. 1). The perilymph peak values were obtained at 30 min after application in all 3 groups and then decreased with time. Peak perilymph concentration was 4.3 µg/ml in the LD group and 18.8 µg/ml in the HD group. They were 0.27 and 0.15%, respectively, of the administered concentrations, which were 1.6 and 12.8 mg/ml. Caroverine became undetectable at 6 h in the LD group, while the concentration still remained at 1.9 µg/ml at 6 h in the HD group. Perilymph caroverine could not be detected in the IV group at 3 h after administration.

As shown in figure 2, caroverine in the CSF was detected in both local administration groups at 30 min after application and remained at very low levels until it became undetectable at 6 h. In the IV group, CSF caroverine reached a much higher peak concentration 10 min after administration and then decreased with time. There was no statistically significant difference ($p = 0.39$) in caroverine concentrations between the perilymph and CSF following IV administration. However, the concentration in the perilymph seemed to be relatively higher than in the CSF.

The concentration of caroverine in plasma (fig. 3) reached a peak value 10 min after both local and intravenous administration of caroverine and then decreased with time. However, caroverine was still detectable 6 h after intravenous administration. In both LD and HD groups, the concentrations were much lower than those in the IV group and became undetectable at 6 h in the LD group.

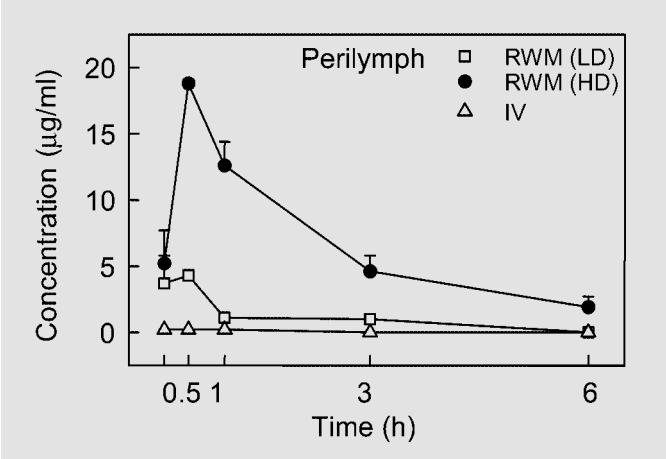


Fig. 1. The pharmacokinetic curves of caroverine in the perilymph after intravenous injection or RWM administrations.

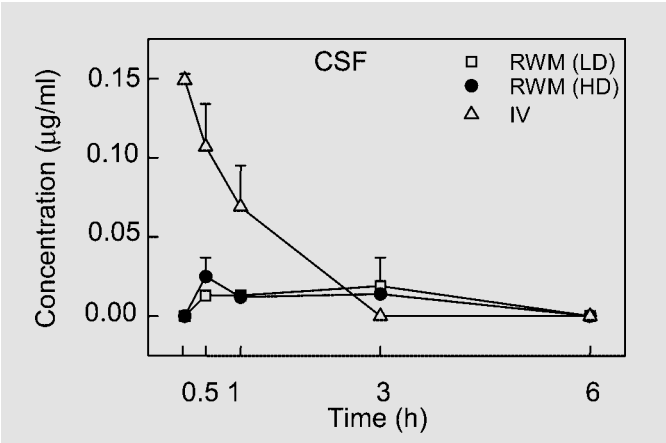


Fig. 2. The pharmacokinetic curves of caroverine in CSF after intravenous injection or RWM administrations.

Auditory Brainstem Responses

ABR thresholds at 4 different frequencies (20, 16, 12.5 and 8 kHz) are shown in figure 4. At 20 kHz, the threshold shifts at 30 min following RWM application were 8, 27 and 56 dB in the control, LD and HD groups, respectively. When comparing ABR threshold values after treatment with the pretreatment values at 20 kHz, both LD and HD groups exhibited significantly impaired thresholds at 30 min, 3 and 6 h after application ($p = 0.009$, 0.001, 0.006 and $p = 0.001$, 0.001, 0.002, respectively). Comparison of ABR threshold shifts at 20 kHz in the LD and HD groups with the control group showed statistically significant differences at 30 min and 3 h ($p = 0.014$ and 0.002 for LD; $p = 0.0003$ and 0.003 for HD). The threshold shift was smaller at 16 and 12.5 kHz and the least at 8 kHz. All the thresholds recovered partially at 3 and 6 h and had completely returned to the normal level 24 h after caroverine application.

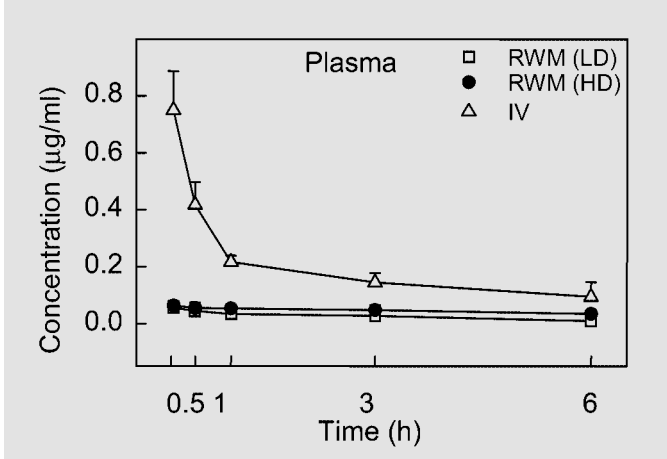


Fig. 3. The pharmacokinetic curves of caroverine in plasma after intravenous injection or RWM administrations.

Wave I of the ABR response is dominated by the cochlear region. To further explore the functional effects, the amplitude and latency of wave I were analyzed at 90 dB at all 4 frequencies (fig. 5, 6). A statistically significant decrease in the wave I amplitude appeared at 20 kHz at 30 min in both the LD and HD groups compared to the control group ($p = 0.0004$ and $p = 0.026$, respectively). The wave I amplitude in the LD group showed less reduction than in the HD group. The amplitude partially recovered at 3 and 6 h and had returned to normal levels at 24 h following application.

The wave I latencies in the HD group were more severely changed than those in the LD group at all 4 frequencies. When compared with the control group, significant latency prolongations were observed at 20 and 12.5 kHz at 30 min in the HD group ($p = 0.007$ and $p = 0.03$, respectively). The latencies in all 3 groups recovered partially at 3 and 6 h and recovered at 24 h after application.

Discussion

There is growing interest in inner ear medication by local routes instead of systemic application, in order to achieve therapeutic levels in the inner ear while avoiding

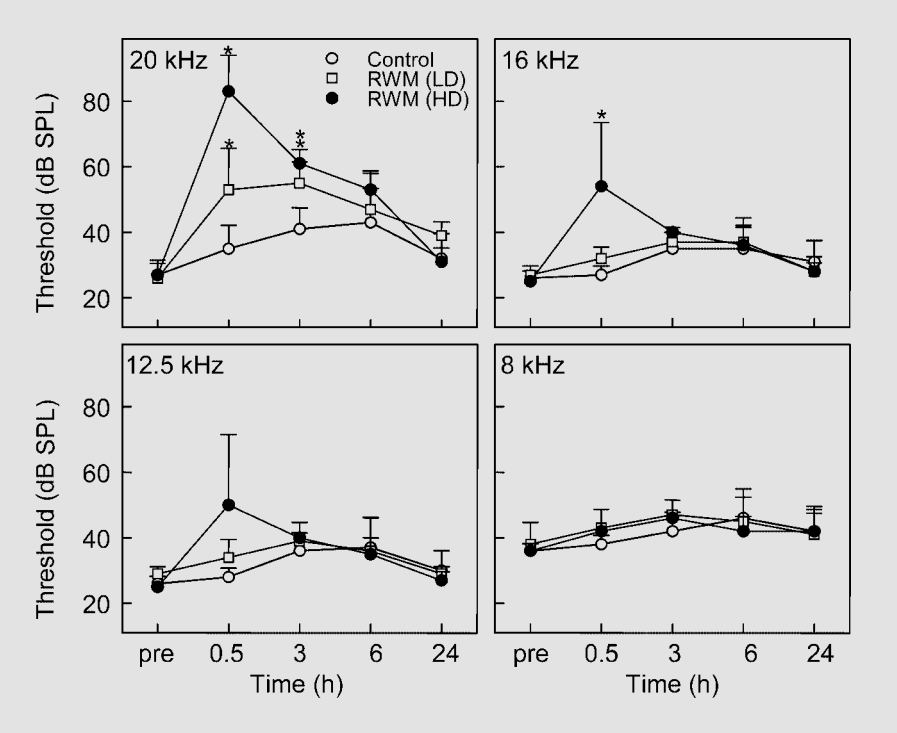


Fig. 4. ABR threshold (dB SPL) with time at 20, 16, 12.5 and 8 kHz. * $p < 0.05$: statistically significant threshold shift compared with the control group.

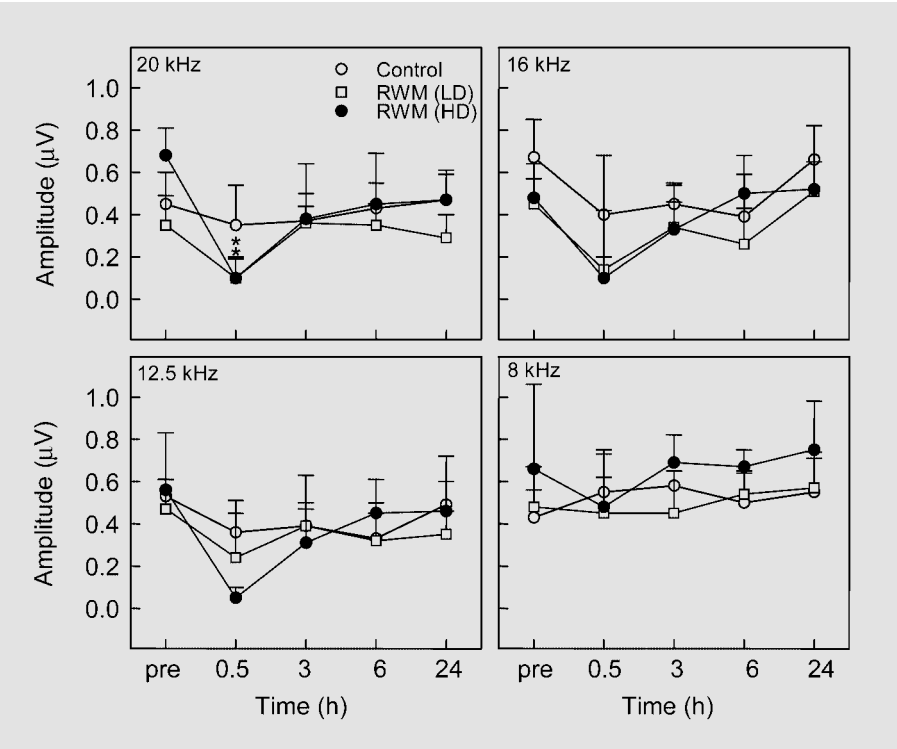


Fig. 5. Wave I amplitude (µV) at 90 dB with time at 20, 16, 12.5 and 8 kHz. * $p < 0.05$: statistically significant amplitude decrease compared with the control group.

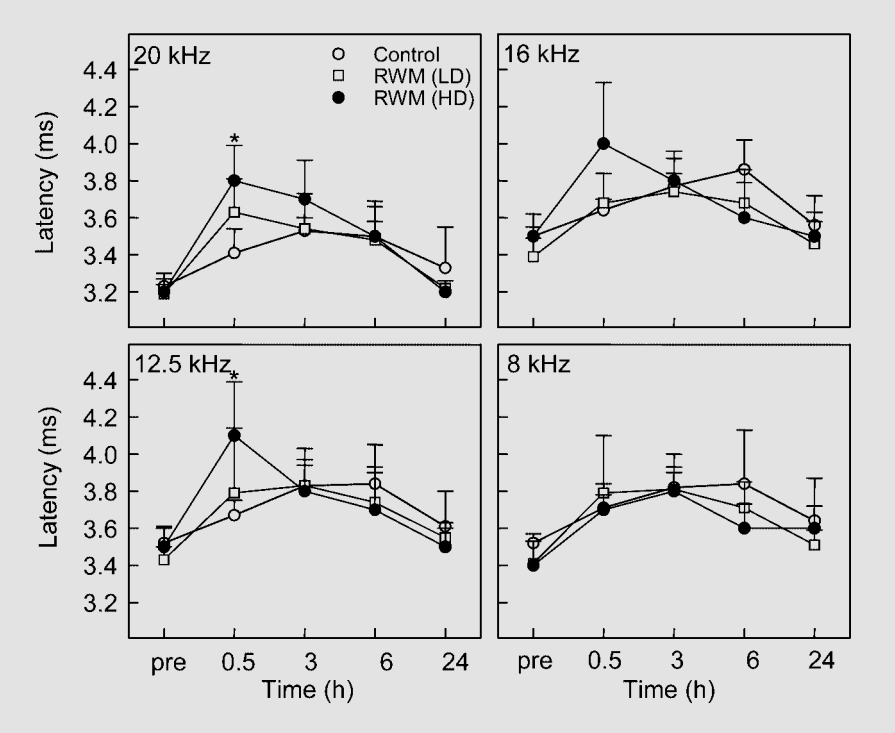


Fig. 6. Wave I latency (ms) at 90 dB with time at 20, 16, 12.5 and 8 kHz. * $p < 0.05$: statistically significant latency prolongation compared with the control group.

undesirable systemic side effects. Caroverine, as a glutamate receptor antagonist, is clinically used for the treatment of tinnitus [Denk et al., 1997]. However, the risk of inducing unwanted side effects appears in most glutamate receptor antagonists if given in large enough doses. Low doses are associated with altered sensory perception, dysphoria, hypertension, nystagmus and disorientation, with progression to agitation, paranoia, hallucinations, severe motor retardation and ultimately catatonia at higher doses [Ehrenberger and Felix, 1992]. These potentially detrimental adverse effects obviously limit their clinical use for treatment of inner ear diseases by systemic administration. Thus it is important to find alternative ways to apply the drug.

Delivery of agents into the inner ear via the RWM is being increasingly used clinically. For example, this approach has been utilized for the delivery of steroids and gentamicin to the inner ear in the treatment of autoimmune diseases, sensorineural hearing loss, tinnitus and Ménière's disease [Silverstein et al., 1996, 1999; Blackley, 1997; Parnes et al., 1999; Hoffer et al., 2001; Schoendorf et al., 2001]. Practically, this is done by insertion of a microcatheter system directly onto the RWM, which results in a more controlled application of the drugs [Schoendorf et al., 2001]. An alternative method is to instill the drugs via the tympanic membrane directly into the middle ear using gelfoam on the RWM as a form of continuous-release vehicle which allows for prolonged drug perfusion of the labyrinth. This method is a relatively simple and effective procedure that has been used in both experimental and clinical studies [Silverstein et al., 1996, 1999].

The present study shows that after local applications of caroverine onto the RWM with gelfoam, the perilymph concentrations quickly reach high peak values at 30 min, followed by a relatively fast decrease over time. The perilymph peak values in the LD and HD RWM groups were almost 20 and 80 times higher than the peak value reached in the IV group. As expected, the high dose produced higher perilymph levels than the low dose, which suggests that the absorption of caroverine through the RWM is a dose-dependent process. The perilymph caroverine concentration fell more slowly in the animals given the drug directly onto the RWM than in those animals given a much higher dose systemically. In the perilymph, caroverine might be removed not only by passive diffusion, but also by active elimination such as blood flow and lymphatic flow [Hibi et al., 2001]. It is possible that the elimination of caroverine is faster than what is shown in our results. The maintenance of the concentration of caroverine in the perilymph is most likely due to the continuous absorption from the gelfoam through the RWM.

It is well known that the RWM is permeable to various drugs and substances placed in the niche area. These include antibiotics, antiseptics, arachidonic acid metabolites, local anesthetics, toxins and albumin [Goycoolea and Lundman, 1997], showing that not only small molecules but also macromolecules can pass through the RWM. Caroverine is a low-molecular-weight substance (molecular weight of caroverine hydrochloride = 420) and should pass through the RWM quite freely. The concentrations of caroverine in the perilymph after local applications were considerably higher than those found following systemic application. The results clearly show the permeability of the RWM to caroverine. Consequently, high concentrations of caroverine can be attained in the perilymph by application of a small amount of the drug on the RWM.

After systemic administration, the concentrations of caroverine in plasma were consistently higher than in CSF and perilymph. This can be attributed to the existences of both blood-brain and blood-labyrinth barriers. The perilymph levels seemed to be higher than CSF concentrations, but the results were not statistically significant. This may reflect the differences between the blood-brain barrier and blood-labyrinth barrier, or the properties of the communication between CSF and perilymph through the cochlear aqueduct. Another important observation, although not entirely unexpected, was that the local caroverine applications resulted in much lower drug concentrations in plasma and CSF as compared to systemic administration. For example, the plasma and CSF caroverine peak values in the IV group were about 12 and 6 times the peak values in the HD group, respectively. A less systemic adverse effect may be expected with the lower caroverine concentrations in plasma and CSF. This is in part related to the dose. The total volume of perilymph is only around 15.9 mm³ [Shinomori et al., 2001]. Therein lies the major advantage of the RWM administration, which is the ability to achieve a high local drug concentration without high blood and CSF levels.

The ideal concentration of caroverine in the perilymph for the treatment of inner ear diseases, such as cochlear synaptic tinnitus, remains unknown. In this study, the intravenous dose used in guinea pigs is the same as that used clinically in the treatment of tinnitus. Assuming similar pharmacokinetics in guinea pigs and humans, one may presume the therapeutic concentration of caroverine to be around 0.2–0.3 μg/ml in the perilymph. The two dosages for RWM application were chosen somewhat arbitrarily. The main purpose of the study was not to establish a therapeutic window but to relate the auditory

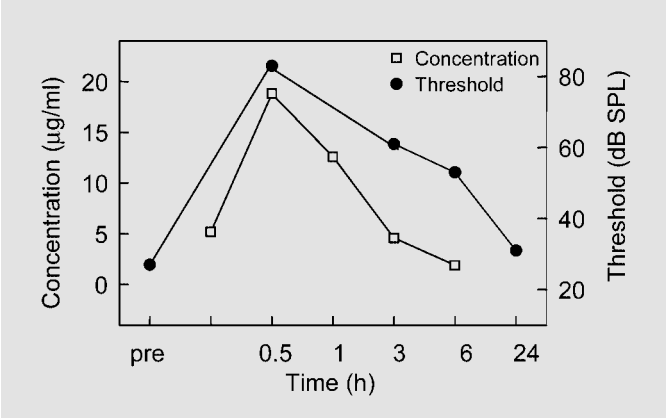


Fig. 7. Perilymph caroverine mean concentrations and thresholds at 20 kHz with time after high-dose local application.

effects to caroverine concentrations in the perilymph. The effects of caroverine on hearing thresholds were tested by measuring the ABR to sound stimuli. The perilymph caroverine concentration is expected to be higher in the basal, high-frequency region of the cochlea, being close to the RWM. Indeed, the maximum changes in hearing threshold, latency and amplitude occurred at 20 kHz at 30 min after application in both local administration groups, and the changes were all statistically significant. The ABR was less affected at the frequencies of 16, 12.5 and 8 kHz, most likely due to the lower caroverine concentrations at positions further from the RWM. The ABR thresholds improved partially at 3 and 6 h and were completely back to normal levels 24 h after administration. The two local dosages caused transient, but reversible hearing dysfunction. As the transient dysfunction is dose related, one would expect it to be negligible at the assumed therapeutic concentration which is much lower than the perilymph concentration after RWM application in the study. The slight hearing impairment seen in the control group was most likely due to the weight of gelfoam and saline on the RWM, surgical stress and possibly an altered ionic balance as sodium and chloride ions will enter the perilymph when saline is applied on the RWM [Molinari, 1972; Colletti et al., 1986; Hisashi et al., 1999].

By combining the pharmacokinetic observations with the changes seen in hearing thresholds in the HD group, it is clearly demonstrated that the ABR effects were related to the concentration of caroverine in the perilymph. Figure 7 shows both the caroverine perilymph concentrations and the ABR threshold (at 20 kHz) as functions of time following a higher-dose RWM administration. At 30 min, when perilymph caroverine reached its peak val-

ue, the ABR threshold shift was also greatest. At 3 and 6 h, when the perilymph caroverine concentration decreased markedly, the hearing threshold recovered partially.

Further studies are necessary to find out the ideal dose and administration paradigm. The study of the effect following local application of caroverine on the RWM in the treatment of excitotoxicity-related inner ear diseases, such as noise-induced hearing loss, can be carried out on the animal models. This information will be useful for the establishment and formulation of the local application method in the clinic in the future.

Acknowledgements

The authors thank Phafag AG, Schaanwald, Liechtenstein, for their supply of caroverine, Y.M. Khoo for technical assistance and Dr. J. Bruton for helpful comments. This study, conducted through a collaboration programme between the National University of Singapore and Karolinska Institutet, was supported by grants from the National Medical Research Council, Singapore, the Swedish Research Council, the Swedish Council for Working Life and Social Research, Karolinska Institutet, AMF Sjukförsäkringsaktiebolag, Stiftelsen Clas Groschinskys Minnesfond, Stiftelsen Lars Hiertas Minne, the Petrus, Augusta Hedlund Foundation and the Foundation Tysta Skolan.

References

Altschuler RA, Sheridan CE, Horn JW, Wenthold RJ: Immunocytochemical localization of glutamate immunoreactivity in the guinea pig cochlea. Hear Res 1989;42:167–174.

Blackley BW: Clinical forum: A review of intratympanic therapy. Am J Otol 1997;18:520–526.

Bullock R: Strategies for neuroprotection with glutamate antagonists. Ann NY Acad Sci 1995; 765:272–278, 298.

Colletti V, Sittoni V, Shaddock LC: An experimental study of inner ear pathology due to NaCl on the round window membrane. Acta Otolaryngol (Stockh) 1986;101:53–58.

Denk DM, Heinzl H, Franz P, Ehrenberger K: Caroverine in tinnitus treatment: A placebo-controlled blind study. Acta Otolaryngol (Stockh) 1997;117:825–830.

Duan ML, Agerman K, Ernfors P, Canlon B: Complementary roles of neurotrophin 3 and an N-methyl-*D*-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. Proc Natl Acad Sci USA 2000;97: 7597–7602.

Duan ML, Ulfendahl M, Laurell G, Counter AS, Pyykkö I, Borg E, Rosenhall U: Protection and treatment of sensorineural hearing disorders caused by exogenous factors: Experimental findings and potential clinical application. Hear Res 2002;169:169–178.

Ehrenberger K, Felix D: Caroverine depresses the activity of cochlear glutamate receptors in guinea pigs: In vivo model for drug-induced neuroprotection? Neuropharmacology 1992; 31:1259–1263.

Ehrenberger K, Felix D: Receptor pharmacological modes for inner ear therapies with emphasis on glutamate receptors: A survey. Acta Otolaryngol (Stockh) 1995;115:236–240.

Eybalin M: Neurotransmitters and neuromodulators in the mammalian cochlea. Physiol Rev 1993;73:309–373.

Eybalin M, Pujol R: Cochlear neuroactive substances. Arch Otorhinolaryngol 1989;246:228–234.

Felix D, Ehrenberger K: A microiontophoretic study of the role of excitatory amino acids at the afferent synapses of mammalian inner ear hair cells. Eur Arch Otorhinolaryngol 1990; 248:1–3.

Glowatzki E, Fuchs PA: Transmitter release at the hair cell ribbon synapse. Nat Neurosci 2002;5: 147–154.

Goycoolea MV, Lundman L: Round window membrane – Structure function and permeability: A review. Microsc Res Tech 1997;36:201–211.

Hibi T, Suzuki T, Nakashima T: Perilymphatic concentration of gentamicin administration intratympanically in guinea pigs. Acta Otolaryngol (Stockh) 2001;121:336–341.

Hisashi K, Komune S, Nakagawa T, Kimitsuki T, Komiyama S: Regulation of inner ear fluid in the guinea pig cochlea after the application of saturated NaCl solution to the round window membrane. Eur Arch Otorhinolaryngol 1999; 256:S2–S5.

Hoffer ME, Allen K, Kopke RD, Weisskopf P, Gottshall K, Wester D: Transtympanic versus sustained-release administration of gentamicin: Kinetics, morphology, and function. Laryngoscope 2001;111:1343–1357.

Koppi S, Eberhardt G, Haller R, König P: Calcium-channel-blocking agent in the treatment of acute alcohol-withdrawal – Caroverine verus meprobamate in a randomized double blind study. Neuropsychobiology 1987;17:49–52.

Lipton SA, Rosenberg PA: Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med 1994;330:613–622.

Matsubara A, Laake JH, Davanger S, Usami S, Ottersen OP: Organization of AMPA receptor subunits at a glutamate synapse: A quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. J Neurosci 1996;16: 4457–4467.

Molinari GA: Alteration of inner ear mechanisms resulting from application of sodium chloride to the round window membrane. Ann Otol Rhinol Laryngol 1972;81:315–322.

Muir KW, Lees KR: Clinical experience with excitatory amino acid antagonist drugs. Stroke 1995;26:503–513.

Niedzielski AS, Wenthold RJ: Expression of AMPA, kainate, and NMDA receptor subunits in cochlear and vestibular ganglia. J Neurosci 1995;15:2338–2353.

Parnes LS, Sun AH, Freeman DJ: Corticosteroid pharmacokinetics in the inner ear fluids: An animal study followed by clinical application. Laryngoscope 1999;109:1–17.

Puel J-L: Chemical synaptic transmission in the cochlea. Prog Neurobiol 1995;47:449–476.

Pujol R, Puel J-L, D'Aldin CG, Eybalin M: Pathophysiology of the glutamatergic synapses in the cochlea. Acta Otolaryngol (Stockh) 1993;113: 330–334.

Ruel J, Chen C, Pujol R, Bobbin RP, Puel JL: AMPA-preferring glutamate receptors in cochlear physiology of adult guinea-pig. J Physiol 1999;518:667–680.

Ryan AF, Brumm D, Kraft M: Occurrence and distribution of non-NMDA glutamate receptor mRNAs in the cochlea. Neuroreport 1991;2: 643–646.

Saletu B, Grunberger J, Anderer P, Linzmayer L, König P: Acute central effects of the calcium channel blocker and antiglutamatergic drug caroverine. Arzneimittelforschung/Drug Res 1995;45:217–229.

Schoendorf J, Neugebauer P, Michel O: Continuous intratympanic infusion of gentamicin via a microcatheter in Ménière's disease. Otolaryngol Head Neck Surg 2001;124:203–207.

Shinomori Y, Spack DS, Jones DD, Kimura RS: Volumetric and dimensional analysis of the guinea pig inner ear. Ann Otol Rhinol Laryngol 2001;110:91–98.

Silverstein H, Arruda J, Rosenberg SI, Deems D, Hester TO: Direct round window membrane application of gentamicin in the treatment of Ménière's disease. Otolaryngol Head Neck Surg 1999;120:649–655.

Silverstein H, Choo D, Rosenberg S, Kuhn J, Seidman M, Stein I: Intratympanic steroid treatment of inner ear disease and tinnitus. Ear Nose Throat J 1996;75:468–488.

Usami S, Matsubara A, Fujita S, Shinkawa H, Hayashi M: NMDA (NMDAR1) and AMPA-type (GluR2/3) receptor subunits are expressed in the inner ear. Neuroreport 1995;6:1161–1164.

Acta Otolaryngol 2003; 123: 905–909

Taylor & Francis
healthsciences

Acute Treatment of Noise Trauma with Local Caroverine Application in the Guinea Pig

ZHIQIANG CHEN[1,2], MATS ULFENDAHL[2], RUNSHENG RUAN[1], LUKE TAN[1] and MAOLI DUAN[2]

From the [1]Department of Otolaryngology, National University of Singapore, Singapore and [2]Department of Clinical Neuroscience and Center for Hearing and Communication Research, Karolinska Institutet, Stockholm, Sweden

Chen Z, Ulfendahl M, Ruan R, Tan L, Duan M. *Acute treatment of noise trauma with local caroverine application in the guinea pig.* Acta Otolaryngol 2003; 123: 905–909.

Intense sound stimulation may result in excessive glutamate release from the inner hair cells, resulting in binding to the post-synaptic glutamate receptors and leading to neuronal degeneration and functional impairment. In this study we investigated the therapeutic effect and time window of caroverine, an N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist, on noise-induced hearing loss. Guinea pigs were exposed to one-third octave band noise centered at 6.3 kHz (110 dB sound pressure limit) for 1 h. One or 24 h after noise exposure, caroverine was applied to the round window membrane. Auditory brainstem responses were recorded at regular time intervals. It was shown that caroverine could significantly decrease hearing impairment after noise trauma when applied 1 but not 24 h after noise exposure. *Key words: auditory function, glutamate receptor antagonist, inner ear, noise-induced hearing loss, round window membrane*

INTRODUCTION

Intense noise stimulation can cause temporary or permanent functional hearing impairment. This functional impairment may be due to mechanical damage of the tissue and/or single cells and/or to metabolic disturbances affecting the cellular physiology (1). It has been demonstrated that one of the main acute effects of high level noise is injury to the dendrites of the primary auditory neurons below the inner hair cells (IHCs) (2, 3). This dendrite damage is mainly due to excessive release of neurotransmitter from the IHCs, which is excitotoxic to the primary auditory neurons (4, 5). Glutamate is the most likely neurotransmitter candidate (6, 7, 25). Three types of ionotropic glutamate receptor have been identified at the post-synaptic nerve endings beneath the IHCs: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (8–11).

A promising approach to prevent glutamate-triggered excitotoxic injury to the afferent neurons would be to block the glutamate receptors (12). Indeed, glutamate receptor antagonists, such as MK-801 and kynurenic acid, have been shown to be effective in the prevention of dendrite damage and the protection of auditory function when applied prior to or during noise exposure (5, 13, 14). An important issue is whether glutamate receptor antagonists are also able to reduce hearing damage when administered after noise exposure.

Caroverine (Spasmium®; Phafag AG), a quinoxaline derivative, has been shown to act as an NMDA and AMPA receptor antagonist on the neurotransmission of IHCs in guinea pigs (15, 16). In a previous study, caroverine was demonstrated to significantly protect auditory function when applied prior to noise exposure (Chen et al., unpublished observation). The aim of this study was to investigate the therapeutic effect and time window of caroverine on noise-induced hearing loss. We administered caroverine locally to the round window membrane (RWM) 1 or 24 h after noise exposure. The auditory brainstem response (ABR) was measured at regular time intervals after RWM application in order to evaluate auditory function.

MATERIAL AND METHODS

Animals and noise exposure

Twenty-four pigmented guinea pigs of either sex (300–400 g) were used in this study. The care and use of the animals were approved by Karolinska Institutet and the local Ethical Committee in Stockholm. The animals were anesthetized by means of i.m. injection with a mixture of ketamine (40 mg/kg) and xylazine (4 mg/kg). The anesthetized animals were exposed to one-third octave band noise centered at 6.3 kHz [110 dB sound pressure limit (SPL)] for 1 h in a soundproof booth. The soundproof booth was equipped with a speaker horn (Model 2328; James B. Lancing Sound Inc., Los Angeles, CA) mounted in the ceiling. The free-field noise exposure was generated using Pulse software (Brüel & Kjær) and delivered by a sound generator (Brüel & Kjær LAN Interface Module type 7533 and Input/Output Module type 3109) connected to an amplifier (Brüel & Kjær type 2716). The noise intensity (110 dB SPL; reference: 20 μPa) was measured prior to exposure using a 0.5-inch microphone (Brüel & Kjær type 4190) and a preamplifier (Brüel & Kjær type 2669C) at the approximate level of the animal's ear.

 ISSN 0001-6498 DOI 10.1080/00016480310000638

Local caroverine or physiological saline application

Twenty-four guinea pigs were randomly divided into four equal groups: one control and one caroverine group in which local applications were performed 1 h after the end of noise exposure, and control and caroverine groups in which local administration occurred 24 h after noise exposure. Under an operating microscope, the right temporal bulla was opened via a post-auricular incision to expose the round window. A small piece of Gelfoam was placed on the RWM. Fifteen μl of either physiological saline or 12.8 mg/ml caroverine solution was applied to the Gelfoam. The hole in the temporal bulla was then closed using dental cement (Fuji I, Japan) and the skin sutured.

ABR measurements

ABR thresholds were obtained 1 day before noise exposure and at regular time intervals (0.5 and 24 h, 3 days and 1 week) following normal saline or caroverine application. The animals were anesthetized as above. ABR measurements were performed in a soundproof booth as described previously (13). Responses were recorded with subcutaneous stainless electrodes as the potential difference between an electrode on the vertex and an electrode behind the ear, with the hind leg serving as the earth. Stimulus intensity was calibrated with a 0.25-inch condenser microphone (Model 4135; Brüel & Kjær). The SPL was expressed as the peak SPL with reference to 20 μPa. The stimulus signals were generated using Tucker-Davis Technologies (Gainesville, FL) equipment controlled by a computer and delivered by a Telephonics TDH 39 earphone (Farmingdale, NY). The stimuli were delivered through a closed acoustic system sealed into the external auditory meatus. The evoked response was amplified 100,000 times and 2048 sweeps were averaged in real time by a digital signal processor (DSP32C) with time-domain artifact rejection. The initial intensity of the stimulus was 100 dB peak SPL; this was then decreased in 10-dB steps until the threshold was approached, and then in 5-dB steps until the ABR disappeared. The threshold was defined as the lowest intensity at which a visible ABR wave was seen in two averaged runs and measured at 5

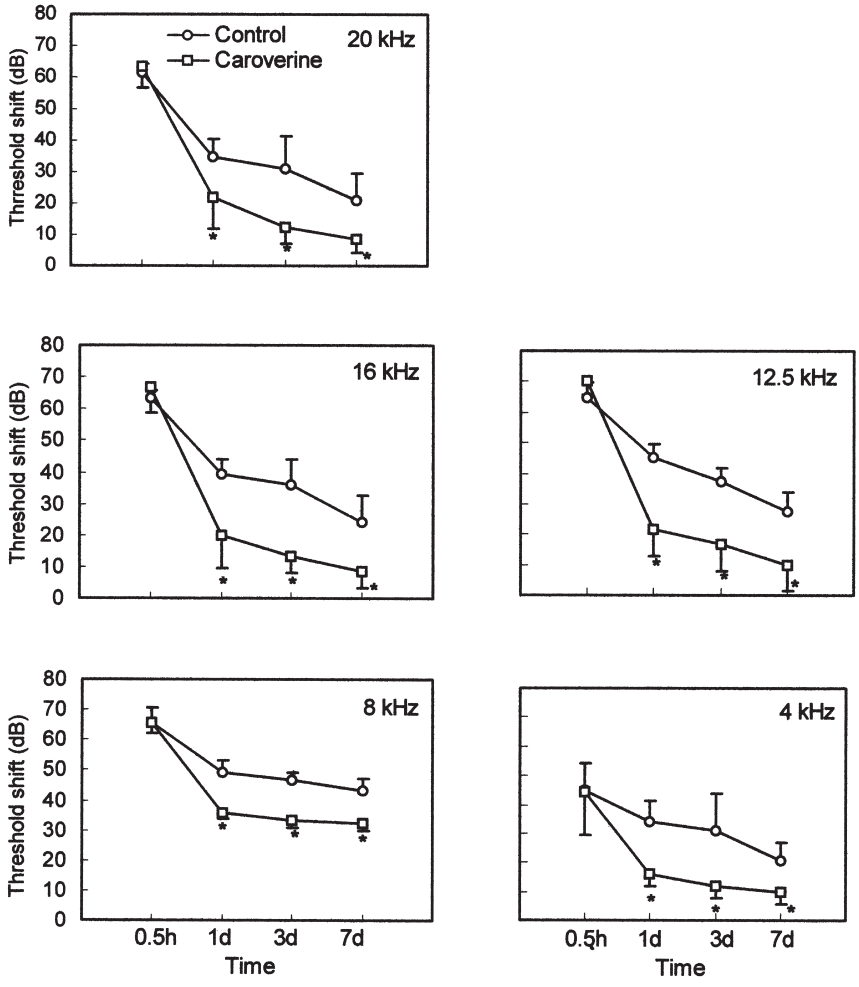


Fig. 1. ABR threshold shifts (mean ± SD) for each tested frequency as a function of time following physiological saline or caroverine application (1 h after noise exposure). * $p < 0.05$ compared with control group.

frequencies: 20, 16, 12.5, 8 and 4 kHz. The differences in mean values of threshold shifts between different groups were tested for significance ($p < 0.05$) using Student's two-tailed t-test.

RESULTS

The effects of caroverine applied 1 h after noise exposure are illustrated in Fig. 1. Half an hour after the RWM applications (1.5 h after noise exposure), both control and caroverine groups showed 60–70 dB threshold shifts at 8–20 kHz and $\approx$45 dB threshold shifts at 4 kHz. The threshold shifts in the control group remained at 35–45 dB at 20, 16, 12.5 and 4 kHz and at 50 dB at 8 kHz, 24 h after RWM application. However, in the caroverine group the threshold shifts decreased to 15–20 dB at 20, 16, 12.5 and 4 kHz and to 35 dB at 8 kHz, 24 h after caroverine application. The threshold shifts in the caroverine group were significantly smaller at all tested frequencies when compared to those in the control group at 24 h, 3 days and 1 week after RWM applications.

The effects of caroverine applied 24 h after noise exposure are illustrated in Fig. 2. The threshold shifts in the control group at 0.5 h after RWM application (24.5 h after noise exposure) were 25–40 dB at all frequencies. In the caroverine group, the threshold shifts were 50–60 dB at 20, 16, 12.5 and 8 kHz and 25 dB at 4 kHz. Twenty-four h after RWM application (48 h after noise exposure), both control and caroverine groups show 15–25 dB threshold shifts at 20, 16, 12.5 and 4 kHz and 40 dB threshold shifts at 8 kHz. No significant difference in threshold shift was found between the control and caroverine groups at 24 h, 3 days and 1 week after RWM application.

DISCUSSION

This study shows that local administration of caroverine 1 h after noise exposure significantly reduces the damage caused to cochlear function after noise trauma. In contrast, treatment with caroverine 24 h after noise exposure failed to achieve any functional protection during the same time period.

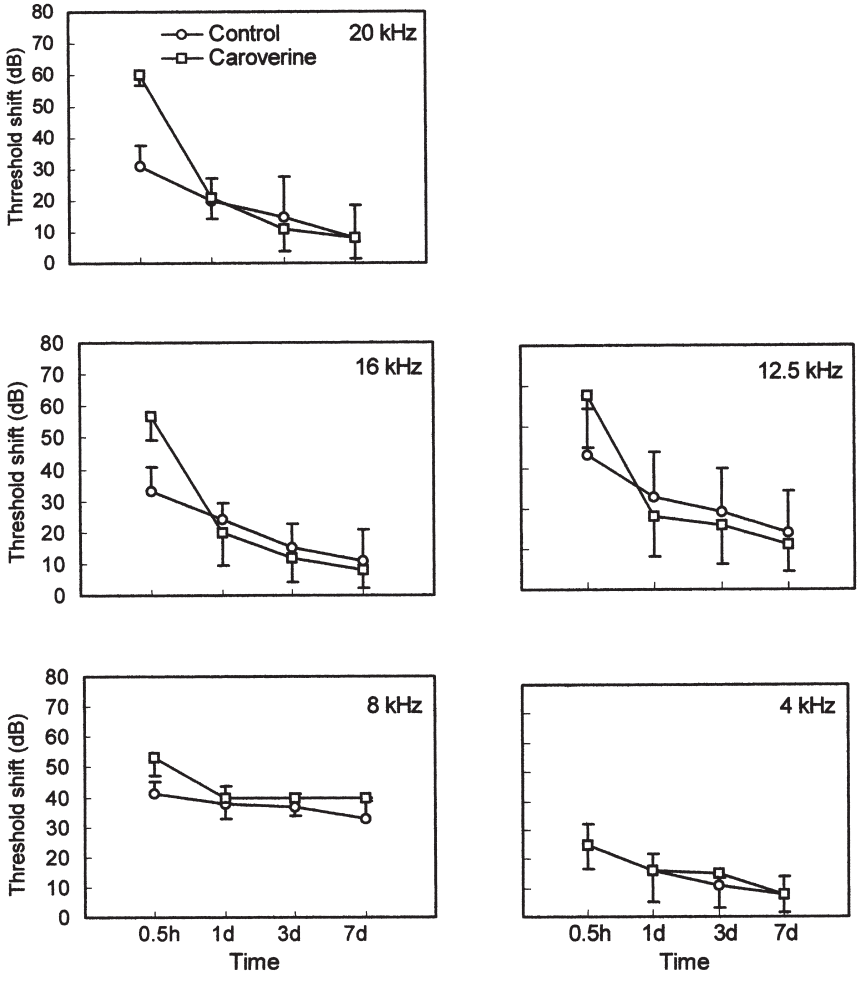


Fig. 2. ABR threshold shifts (mean $\pm$ SD) for each tested frequency as a function of time following physiological saline or caroverine application (24 h after noise exposure).

In a previous study (17), a high concentration of caroverine was detected in the perilymph during the first 6 h following RWM application using the same protocol and dose as in the present study, and the ABR effects were related to the concentration of caroverine in the perilymph. The present results showed no significant decrease in threshold shifts in the caroverine group compared with the control group at 0.5 h after RWM application (1.5 h after noise exposure). This may be due to the high concentration of caroverine in the perilymph, leading to caroverine binding to the glutamate receptors, and thus blocking the effect of the neurotransmitter (glutamate). Interestingly, a significant improvement in auditory function was found at 24 h, 3 days and 1 week after caroverine application.

It appears that non-NMDA receptors are activated by low-to-moderate intensity sound, whereas NMDA receptors are activated by high-intensity stimuli (18). In our experiment, stimulation with 110 dB SPL noise would have activated both NMDA and non-NMDA receptors. It has been suggested that NMDA receptor activation in the presence of excessive glutamate is mainly responsible for the initial disturbance of neuronal ion homeostasis, whilst AMPA/kainate receptors contribute to the development of neuronal damage at a stage when NMDA receptors begin to play a less prominent role (19). Excitotoxic injury to the cochlea may occur during noise trauma, ischemia or other types of energy failure (20). In animal models of cerebral ischemia, NMDA receptor antagonists seem to be effective against ischemic injury only when administered before or shortly after the ischemic insult (21). However, the AMPA/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxaline is effective in reducing damage in rats subjected to global ischemia even when administered several hours after the ischemic insult (22, 23). Talampanel, another AMPA receptor antagonist, has been shown to significantly attenuate neuronal damage when administered 30 min, but not 3 h, after brain trauma in rats (24). The mechanism by which caroverine can attenuate auditory impairment when applied 1 but not 24 h after noise exposure is not fully known. One possible explanation may be that the auditory functional impairment was caused by both metabolic and mechanical damage due to noise trauma. The process of metabolic change may still be in progress 1 h after noise exposure via glutamate release; the excess glutamate may not have been eliminated at this time, but the metabolic process may have ceased 24 h after noise trauma.

In conclusion, this study demonstrates that acute treatment with caroverine is beneficial in noise-induced hearing loss. However, the therapeutic window is narrow.

ACKNOWLEDGEMENTS

The authors thank Phafag AG, F. Liechtenstein, Switzerland for providing caroverine, Professor E. Borg and Dr L. Järlebark for helpful comments and A. Fransson and P. Mannström for technical assistance. This study, conducted through a collaborative program between the National University of Singapore and Karolinska Institutet, was supported by grants from the National Medical Research Council, Singapore, the Swedish Research Council, the Swedish Council for Working Life and Social Research, Karolinska Institutet, the Petrus and Augusta Hedlund Foundation, the Foundation Tysta Skolan and Stiftelsen Clas Groschinskys Minnesfond.

REFERENCES

1. Saunders JC, Dear SP, Schneider ME. The anatomical consequences of acoustic injury: a review and tutorial. J Acoust Soc Am 1985; 78: 833–60.
2. Spoendlin H. Primary structural changes in the organ of Corti after acoustic overstimulation. Acta Otolaryngol (Stockh) 1971; 71: 166–76.
3. Robertson D. Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hear Res 1983; 9: 263–78.
4. Puel JL, d'Aldin C, Safieddine S, Eybalin M, Pujol R. Excitotoxicity and plasticity of IHC-auditory nerve contributes to both temporary and permanent threshold shift. In: Axelsson A, Borchgrevink H, Hamernik RP, Hellstrom PA, Henderson D, Salvi RJ, eds. Scientific basis of noise-induced hearing loss. New York: Thieme; 1996. p. 36–42.
5. Puel JL, Ruel J, Gervais d'Aldin C, Pujol R. Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. Neuroreport 1998; 9: 2109–14.
6. Eybalin M. Neurotransmitter and neuromodulators in the mammalian cochlea. Physiol Rev 1993; 73: 309–73.
7. Puel J-L. Chemical synaptic transmission in the cochlea. Prog Neurobiol 1995; 47: 449–76.
8. Ryan AF, Brumm D, Kraft M. Occurrence and distribution of non-NMDA glutamate receptor mRNAs in the cochlea. Neuroreport 1991; 2: 643–6.
9. Niedzielski AS, Wenthold RJ. Expression of AMPA, kainate, and NMDA receptor subunits in cochlear and vestibular ganglia. J Neurosci 1995; 15: 2338–53.
10. Usami S, Matsubara A, Fujita S, Shinkawa H, Hayashi M. NMDA (NMDAR1) and AMPA-type (GluR2/3) receptor subunits are expressed in the inner ear. Neuroreport 1995; 6: 1161–4.
11. Matsubara A, Laake JH, Davanger S, Usami S, Ottersen OP. Organization of AMPA receptor subunits at a glutamate synapse: a quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. J Neurosci 1996; 16: 4457–67.
12. Duan ML, Ulfendahl M, Laurell G, et al. Protection and treatment of sensorineural hearing disorders caused

by exogenous factors: experimental findings and potential clinical application. Hear Res 2002; 169: 169–78.
13. Duan ML, Agerman K, Ernfors P, Canlon B. Complementary roles of neurotrophin 3 and a N-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. Proc Natl Acad Sci U S A 2000; 97: 7597–602.
14. Chen GD, Kong J, Reinhard K, Fechter LD. NMDA receptor blockage protects against permanent noise-induced hearing loss but not its potentiation by carbon monoxide. Hear Res 2001; 154: 108–15.
15. Ehrenberger K, Felix D. Caroverine depresses the activity of cochlear glutamate receptors in guinea pigs: in vivo model for drug-induced neuroprotection? Neuropharmacology 1992; 31: 1259–63.
16. Oestreicher E, Ehrenberger K, Felix D. Different action of memantine and caroverine on glutamatergic transmission in the mammalian cochlea. Adv Otorhinolaryngol 2002; 59: 18–25.
17. Chen Z, Duan M, Lee H, Ruan R, Ulfendahl M. Pharmacokinetics of caroverine in the inner ear and its effects on cochlear function after systemic and local administrations in guinea pigs. Audiol Neurootol 2003; 8: 49–56.
18. Felix D, Ehrenberger K. N-methyl-D-aspartate-induced oscillations in excitatory afferent neurotransmission in the guinea pig cochlea. Eur Arch Otorhinolaryngol 1991; 248: 429–31.
19. Prehn JH, Lippert K, Krieglstein J. Are NMDA or AMPA/kainate receptor antagonists more efficacious in the delayed treatment of excitotoxic neuronal injury? Eur J Pharmacol 1995; 292: 179–89.
20. Puel JL, d'Aldin C, Ruel J, Ladrech S, Pujol R. Synaptic repair mechanisms responsible for functional recovery in various cochlear pathologies. Acta Otolaryngol (Stockh) 1997; 117: 214–8.
21. Meldrum B. Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission. Cerebrovasc Brain Metab Rev 1990; 2: 27–57.
22. Sheardown MJ, Nielsen EO, Hansen AJ, Jacobsen P, Honore T. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo-(F)-quinoxaline: a neuroprotectant for cerebral ischemia. Science 1990; 247: 571–4.
23. Li H, Buchan AM. Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. J Cereb Blood Flow Metab 1993; 13: 933–9.
24. Belayev L, Alonso OF, Liu Y, et al. Talampanel, a novel noncompetitive AMPA antagonist, is neuroprotective after traumatic brain injury in rats. J Neurotrauma 2001; 18: 1031–8.
25. Klinke R. Neurotransmission in the inner ear. Hear Res 1986; 22: 235–243.

Submitted November 14, 2002; accepted January 23, 2003

Address for correspondence:
Maoli Duan, MD, PhD
Center for Hearing and Communication Research
Building M1:02
Karolinska Hospital
SE-171 76 Stockholm
Sweden
Tel.: +46 8 51773210
Fax: +46 8 348546
E-mail: maoli.duan@cfh.ki.se

Protection of Auditory Function against Noise Trauma with Local Caroverine Administration in Guinea Pigs

Accepted for publication by Hearing Research

Zhiqiang Chen [a, c], Mats Ulfendahl [b, c, d], Runsheng Ruan [a], Luke Tan [a], Maoli Duan [b, c, d]

[a] Department of Otolaryngology, National University of Singapore, Lower Kent Ridge Road, Singapore

[b] Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

[c] Center for Hearing and Communication Research, Karolinska Institutet, Stockholm, Sweden

[d] Department of Otolaryngology, Karolinska Hospital, Stockholm, Sweden

Address for correspondence: Maoli Duan, MD, PhD
Center for Hearing and Communication Research
Building M1: 02
Karolinska Hospital
SE-171 76 Stockholm, Sweden
Tel.: +46 8 51773210 Fax: +46 8 348546
E-mail: maoli.duan@cfh.ki.se

Abstract

Glutamate is the most likely neurotransmitter at the synapse between the inner hair cell and its afferent neuron in the peripheral auditory system. Intense noise exposure may result in excessive glutamate release, binding to the post-synaptic receptors and leading to neuronal degeneration and hearing impairment. The present study investigated the protective effect of caroverine, an antagonist of two glutamate receptors, N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, on noise-induced hearing loss. Two different doses of caroverine were applied onto the round window membrane with gelfoam, followed by one-third-octave band noise centered at 6.3 kHz (110 dB SPL) for 1 h. Auditory brainstem responses were measured at regular time intervals afterwards. Caroverine was found to offer significant protection of the cochlear function against noise-induced hearing loss.

Key words:

Caroverine; Glutamate receptor antagonist; Protection; Noise-induced hearing loss; Auditory brainstem response; Guinea pig

Introduction

There is abundant evidence that glutamate is the excitatory neurotransmitter in the peripheral auditory system (Klinke and Oertel, 1977; Klinke, 1986; Altschuler et al., 1989; Eybalin, 1993; Puel, 1995; Glowatzki and Fuchs, 2002). Three types of ionotropic glutamate receptors have been shown to be present at the post-synaptic nerve endings beneath the inner hair cells: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) (Ryan et al., 1991; Niedzielski and Wenthold, 1995; Usami et al., 1995; Matsubara et al., 1996). Excessive or prolonged activation of glutamate receptors can lead to neuronal cell death and contribute to a wide spectrum of neurologic disorders. The process is characterized by two main elements: depolarization of neurons with Na+ influx and the entry of extracellular Ca2+ into neuronal cells. Depolarization is primarily initiated by activation of AMPA receptors and subsequently the voltage-dependent Na+ channels. The entry of Na+ is followed by a passive entry of Cl- and water, resulting in an increase in cellular volume and acute neuronal swelling. This osmotic component is potentially reversible if the stimulus is removed (Choi, 1987). If the stimulus remains, the continuous depolarization will release the magnesium blockage of the NMDA receptor, leading to the opening of the NMDA receptor. The elevated extracellular glutamate causes the influx of Ca2+ into neuronal cells through the opened NMDA receptors. Intracellular Ca2+ will also rise due to impaired activity of the membrane Na+/Ca2+ exchanger (Koch and Barish, 1994). The increased intracellular free Ca2+ will stimulate the activity of numerous enzymes and trigger other calcium-dependent protein-protein interactions that are ultimately deleterious to cell homeostasis, and thus will lead to neuronal death (Doble, 1999).

It has been suggested that acoustic overstimulation results in excessive release of glutamate from the inner hair cells, which, by binding to the post-synaptic receptors, causes cellular destruction and neuronal degeneration, thus leading to noise-induced hearing loss (Saunders and Rhyne, 1970; Spoendlin, 1971; Zivin and Choi, 1991, Puel et al., 1994; Shero et al., 1998; Duan et al., 2002). Indeed, application of glutamate agonists has been shown to induce destruction of primary auditory dendrites and to alter cochlear function in a fashion similar to that observed after acoustic trauma (Spoendlin, 1971; Robertson, 1983; Pujol et al., 1985; Duan and Canlon, 1996). Moreover, significant glutamate efflux has recently been demonstrated in the cochlea under intense noise stimulation both in vitro and in vivo (Bledsoe et al., 1980; Jäger et al., 1998, 2000). Thus, it should be expected that a glutamate receptor antagonist would protect cochlear function against noise trauma if the dendritic damage is caused by an excessive release of glutamate from inner hair cells.

Caroverine (Spasmium®, Phafag AG), a quinoxaline-derivative, clinically available as a spasmolytic drug, has been demonstrated to act as a specific, but reversible antagonist of NMDA and AMPA receptors in the cochlear afferents in guinea pigs (Ehrenberger and Felix, 1992; Oestreicher et al., 2002). Clinically, it has been

shown to be effective in the treatment of cochlear synaptic tinnitus (Denk et al., 1997). Unfortunately, most glutamate receptor antagonists have intolerable neuropsychiatric effects and thus cannot be administered systemically (Lipton and Rosenberg, 1994; Bullock, 1995; Muir and Lees, 1995). In a previous study, local administration of caroverine onto the round window membrane (RWM) was found to significantly increase the perilymph caroverine concentration compared to that following systemic application (Chen et al., 2003). The present study used this local application method to test whether caroverine could decrease the noise-induced hearing loss in the guinea pig, and furthermore, to test the hypothesis that excessive glutamate is released from the inner hair cells to the synapse leading to hearing impairment following noise exposure.

Materials and Methods

Animals and local RWM administration

Pigmented guinea pigs of either sex (300 - 400 g) were used. The animals were anesthetized with a mixture of ketamine (40 mg/kg) and xylazine (4 mg/kg) through intramuscular injection and additional anesthesia was added when necessary. Under an operating microscope, the right temporal bulla was opened through a post-auricular incision to expose the round window under aseptic conditions. The RWM was examined under microscopy to make sure that the RWM was clean and intact before drug administration. The round window membrane was seldom damaged by the surgery. We discarded the animals when we found the round window membrane was not intact. A small piece of gelfoam was placed on the RWM. Fifteen microlitres of either physiological saline or caroverine at two different concentrations were dropped onto the gelfoam. Eighteen animals were randomly divided into 3 groups with 6 animals in each group. The control group received 15 µl of physiological saline, the low dose group (LD) received 15 µl of 1.6 mg/ml of caroverine in normal saline, and the high dose group (HD) 15 µl of 12.8 mg/ml of caroverine in normal saline. The hole of the temporal bulla was then closed using dental cement (Fuji I, Japan) and the skin sutured. After the terminal ABR measurement, the animal was decapitated after giving an overdose of pentobarbital and the bulla was removed from the skull and opened to examine the middle ear and the round window under microscopy. Then the cochlea was put into 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and a small hole was made into the cochlear apex in order to examine if there was any damage, which could not be observed under the operating microscope. A plastic pipette was used to perfuse the cochlea with 4% paraformaldehyde gently from the opening in the cochlear apex so that any small hole on the round window membrane could be found under microscope. No obvious sign of inflammation was found in the middle ear or round window. The care for and use of the animals were approved by the Ethical Committee at the Karolinska Institutet in Stockholm.

Noise exposure

Ten minutes after the administration of either physiological saline or caroverine, the anesthetized animals were transferred to a sound proof booth and were exposed to one-third octave band noise centered at 6.3 kHz (110 dB SPL) for 1 h. Normally one administration of anesthesia will last for more than one hour in the guinea pig. So it is not necessary to give additional anesthesia for the one-hour noise exposure. The sound proof booth was equipped with a speaker horn (model 2328, James B. Lancing Sound Inc, Los Angeles, CA) mounted in the ceiling. The free field noise exposure was generated with software from Brüel & Kjær (Pulse) and delivered by a sound generator (Brüel & Kjær LAN Interface Module type 7533, Input/Output Module type 3109) connected to an amplifier (Brüel & Kjær type 2716). The noise intensity (110 dB) was measured prior to exposures using a ½ inch microphone (Brüel & Kjær type 4190) and a preamplifier (Brüel & Kjær type 2669C) at the approximate level of the animal's ear.

ABR measurements

ABR thresholds were obtained 1 day before noise exposure and at 1.5 h (20 min after noise exposure), 3, 6, 24 h, 3 days and 1 week after local RWM applications. ABR measurements were performed in a sound proof booth as described previously (Duan et al., 2000). The animals were anesthetized as above before every ABR measurement and the body temperature was maintained at 38°C by using an isothermic heating pad. Responses were recorded with subcutaneous stainless electrodes as the potential difference between an electrode on the vertex and an electrode on the mastoid, while the leg served as ground. Stimulus intensity was calibrated with a ¼ inch condenser microphone (Brüel & Kjær Instruments, Marlborough, Mass., USA, model 4135) and all sound pressure levels were expressed in dB relative to 20 µPa. The stimulus signal was generated using Tucker-Davis Technologies (Gainesville, Fla., USA) equipment consisting of an array processor card (AP) with DSP32 signal processor, 16 bit AD/DA converter, anti-aliasing filters and program controllable attenuators which was controlled by a personal computer. The stimulus was a sine wave with 1 ms rise/fall time, the duration was 4 ms, and the repetition rate was 20 per second. The duration of the ABR window was 10 ms. The stimuli were delivered by an earphone (Telephonics TDH 39, Farmingdale, N.Y., USA) through a closed acoustic system sealed into the external auditory meatus. The evoked response was amplified 100,000 times and 2,048 sweeps were averaged in real time by a digital signal processor (DSP32C) with a time-domain artificial rejection. The initial intensity of the stimulus was 100 dB peak SPL and was then decreased in 10-dB steps until the threshold was approached, and then in 5-dB steps until the ABR disappeared. Threshold was defined as the lowest intensity at which a visible ABR wave III was seen in 2 averaged runs. Threshold was measured at 5 frequencies: 20, 16, 12.5, 8 and 4 kHz.

Statistics

One-way repeated measures analysis of variance (ANOVA) was used to determine if there was a significant effect of caroverine treatment, followed by Tukey test for significance versus the control group at specific frequencies.

Results

The pre-exposure thresholds are shown in Fig. 1. The thresholds were around 20-30 dB at 20, 16, 12.5 and 4 kHz, and 35 dB at 8 kHz. There was no significant difference between control group, LD group and HD group. ABR threshold shifts, determined by the comparison of the post-exposure thresholds at different time points with the pre-exposure thresholds, are plotted in Fig. 2. All three groups showed threshold shifts ranging from 50 to 70 dB across frequencies at 1.5, 3 and 6 h after RWM applications, irrespective of whether it was from control or caroverine treatment group. At 24 h after local application, the control group showed a recovery of around 20 dB at all tested frequencies. For the caroverine groups, however, the recovery was much more pronounced. At 24 h the HD group showed a 40-50 dB threshold recovery at 20, 16, 12.5 and 4 kHz, and about 30 dB recovery at 8 kHz. And the threshold recovery was significantly larger than that for the control group at all 5 frequencies ($p<0.05$). In the LD group, the recovery was smaller but was still significant compared to the control group at 24 h at the two highest frequencies (a 20-35 dB recovery at 20 and 16 kHz; $p = 0.0004$, and $p = 0.007$, respectively).

Further recovery of auditory function was monitored at 3 days and 1 week after local applications. In all the three groups, the threshold recovered around 5 dB at 3 days compared to that at 24 h and also about 5 dB at 1 week compared to that at 3 days. However, the difference in threshold among 24 h, 3 days or 1 week is not significant in each group. When compared to the control group, the HD group showed significant difference in threshold shifts at all 5 frequencies at 3 days after noise exposure, and at 20 and 12.5 kHz at 1 week. In the LD group, threshold shifts of significant difference compared with that in the control group were observed at 20, 16, 8 and 4 kHz at 3 days, and at 20 kHz at 1 week.

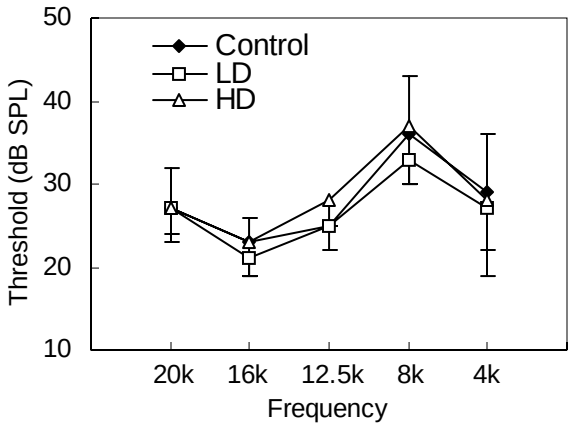


Fig. 1. Pre-exposure ABR threshold across frequencies tested.

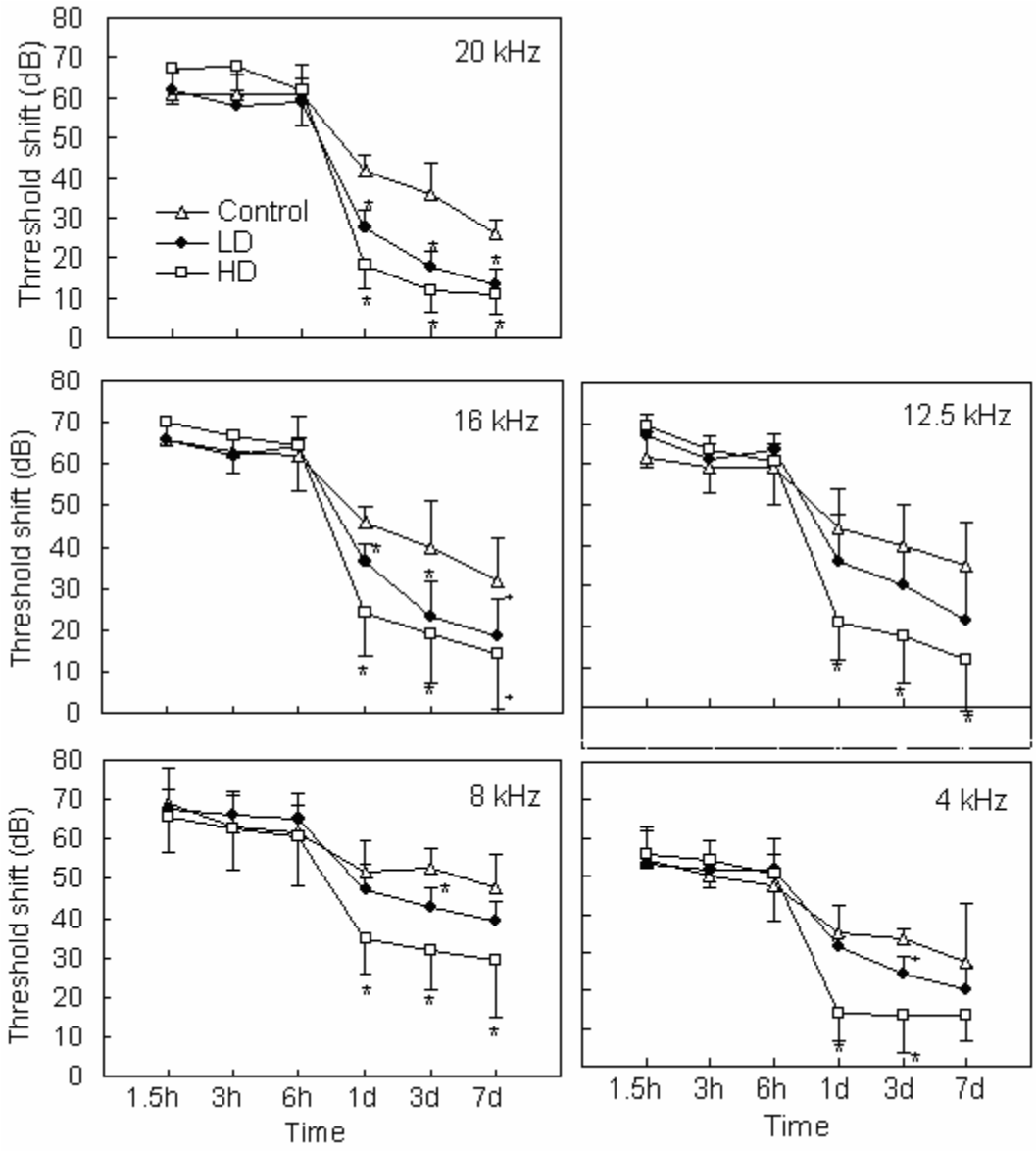


Fig. 2. ABR threshold shifts (mean ± SD) for each tested frequency with time following normal saline or caroverine applications. LD = Low dose group (15 µl of 1.6 mg/ml of caroverine). HD = High dose group (15 µl of 12.8 mg/ml of caroverine).

*: $p<0.05$: statistically significant difference compared with the control group.

Discussion

The present study demonstrates that local caroverine administration directly onto the RWM produced significant protection of cochlear function against noise trauma. The effect was dose-dependent, with greater protective effect in the HD group than in the LD group. Significant protection, as measured using ABR threshold shift, was found at all tested frequencies (4-20 kHz) at 24 h and 3 days and at some frequencies even at 1 week. These results support the notion that pharmacological protection of auditory function has a promising potential for the prevention of noise-induced hearing impairment.

There is accumulating evidence that noise exposure causes excessive release of glutamate from the inner hair cells to the synapse, leading to neuronal degeneration and auditory functional impairment (Spoendlin, 1971; Pujol et al., 1985, 1992; Puel et al., 1994 Jäger et al., 1998, 2000). Thus, glutamate receptor antagonists will block the receptors and might have protective effects against noise. Indeed, glutamate receptor antagonists, such as MK-801 and kynurenic acid, have been shown to be effective in the protection of neuronal dendrite damage against noise trauma and consequently preserve hearing (Puel et al., 1998; Duan et al., 2000; Chen et al., 2001; Ohinata et al., 2003). During noise exposure, it appears that non-NMDA receptors are activated by low-to-moderate stimulus intensities, whereas NMDA receptors are activated by high-intensity sounds (Felix and Ehrenberger, 1991). In our experiments, noise exposure at 110 dB SPL would have activated both NMDA and non-NMDA receptors. As an NMDA and AMPA receptor antagonist, caroverine would block the two receptors when administrated onto the RWM, and thus prevent the excitotoxicity and preserve hearing.

The present study demonstrates that caroverine can effectively protect the auditory function against noise trauma when applied immediately prior to noise exposure. Within the first 6 h after RWM application, there was no significant difference in threshold shift between the LD or HD group and control group. This is most likely caused by high concentration of caroverine in the perilymph, which binds to the glutamate receptors and thus blocks the effect of the neurotransmitter (glutamate) and the sound transduction. In a previous study, it was shown that caroverine readily permeated the RWM in the guinea pig when applied locally onto the RWM (Chen et al., 2003). Caroverine concentration in the perilymph reached its peak value at 30 min after both low and high dose local applications and then decreased with time. Peak perilymph concentration was 4.3 μg/ml in the LD group, and 18.8 μg/ml in the HD group. Caroverine became undetectable at 6 h in the LD group, while the concentration still remained at 1.9 μg/ml at 6 h in the HD group. In the control group, the effect of surgery on the ABR was small and completely disappeared at 24 h after operation. In the caroverine groups, the effect on hearing was mainly seen at the higher frequencies (i.e., closer to the round window). At 30 min after caroverine application there was a 56 dB threshold shift at 20 kHz and only 6 dB threshold shift at 8 kHz in the HD group, which recovered partially at 3

and 6 h. Thresholds became normal at 24 h. In the present study, it is difficult to determine how much of the ABR threshold shift in the first 24 h after noise exposure is a noise affect and how much a drug affect. However, it is certain that the threshold shift is partially caused by caroverine based on the previous study. This might imply the mechanism of protective effect of carvorine against noise. High concentration of caroverine in the cochlear fluid will block the glutamate receptors, which might prevent the cochlear damage caused by excessive glutamate after noise exposure. Of course, this is in the cost of decreased physiological neurotransmission in the cochlea. However, without this kind of block to neurotransmitter, the metabolic damage via over-release of glutamate by noise will continue and consequently cause hearing loss, which can be clearly seen in control noise exposure group. The protective effect was found at 24 h after noise exposure because the effect of caroverine on ABR threshold was completely eliminated at 24 h after local application, which was demonstrated in the previous study. The protection was still manifest at some frequencies 1 week after noise exposure.

In conclusion, the results of this study demonstrate that caroverine can significantly protect the cochlea from noise trauma. The protective effect of caroverine, an NMDA and AMPA receptor antagonist, against noise trauma supports the notion that the excessive release of glutamate from the inner hair cells plays an important role in the pathophysiology of noise-induced hearing loss. Thus, pharmacological protection of the cochlea against noise is possible and may be of great clinical potential.

Acknowledgements

The authors thank Phafag AG, Schaanwald, Liechtenstein for their supply of caroverine, Professor Åke Flock, Drs J. Bruton, L. Järlebark, A.C. Johnson, J. Boutet de Monvel for helpful comments. This study, conducted through a collaboration programme between the National University of Singapore and Karolinska Institutet, was supported by grants from National Medical Research Council, Singapore, the Swedish Research Council, the Swedish Council for Working Life and Social Research, Karolinska Institutet, the Petrus and Augusta Hedlund Foundation, the Foundation Tysta Skolan, and Stiftelsen Clas Groschinskys Minnesfond.

References

Altschuler, R.A., Sheridan, C.E., Horn, J.W., Wenthold, R.J., 1989. Immunocytochemical localization of glutamate immunoreactivity in the guinea pig cochlea. Hear. Res. 42, 167-173.

Bledsoe, S.C. Jr., Bobbin, R.P., Thalmann, R., Thalmann, I., 1980. Stimulus-induced release of endogenous amino acids from skins containing the lateral-line organ in Xenopus laevis. Exp. Brain Res. 40, 97-101.

Bullock, R., 1995. Strategies for neuroprotection with glutamate antagonists. Ann. N. Y. Acad. Sci. 765, 272-278, 298.

Chen, G.D., Kong, J., Reinhard, K., Fechter, L.D., 2001. NMDA receptor blockage protects against permanent noise-induced hearing loss but not its potentiation by carbon monoxide. Hear. Res. 154, 108-115.

Chen, Z., Duan, M., Lee, H., Ruan, R., Ulfendahl, M., 2003. Pharmacokinetics of caroverine in the inner ear and its effects on cochlear function after systemic and local administrations in guinea pigs. Audiol. Neurootol. 8, 46-58.

Choi, DW., 1987. Ionic dependence of glutamate neurotoxicity. J. Neurosci. 7, 369-379.

Denk, D.M., Heinzl, H., Franz, P., Ehrenberger, K., 1997. Caroverine in tinnitus treatment. A placebo-controlled blind study. Acta Otolaryngol. (Stockh.) 117, 825-830.

Doble, A., 1999. The role of excitotoxicity in neurodegenerative disease: implications for therapy. Pharmacol. Ther. 81, 163-221.

Duan, M.L., Agerman, K., Ernfors, P., Canlon, B., 2000. Complementary roles of neurotrophin 3 and a N-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. Proc. Natl. Acad. Sci. USA. 97, 7597-7602.

Duan, M.L., Canlon, B., 1996. Forward masking is dependent on inner hair cell activity. Audiol. Neurootol. 1, 320-327.

Duan, M.L., Ulfendahl, M., Laurell, G., Counter, A.S., Pyykko, I., Borg, E., Rosenhall, U., 2002. Protection and treatment of sensorineural hearing disorders caused by exogenous factors: experimental findings and potential clinical application. Hear. Res. 169, 169-178.

Ehrenberger, K., Felix, D., 1992. Caroverine depresses the activity of cochlear glutamate receptors in guinea pigs: in vivo model for drug-induced neuroprotection? Neuropharmacol. 31, 1259-1263.

Eybalin, M., 1993. Neurotransmitter and neuromodulators in the mammalian cochlea. Physiol. Rev. 73, 309-373.

Felix, D., Ehrenberger, K., 1991. N-methyl-D-aspartate-induced oscillations in excitatory afferent neurotransmission in the guinea pig cochlea. Eur. Arch. Otorhinolaryngol. 248, 429-431.

Glowatzki, E., Fuchs, P.A., 2002. Transmitter release at the hair cell ribbon synapse. Nature Neurosci. 5, 147-154.

Jäger, W., Goiny, M., Herrera-Marschitz, M., Brundin, L., Fransson, A., Canlon, B., 2000. Noise-induced aspartate and glutamate efflux in the guinea pig cochlea and hearing loss. Exp. Brain Res. 134, 426-434.

Jäger, W., Goiny, M., Herrera-Marschitz, M., Flock, Å., Hökfelt, T., Brundin, L., 1998. Sound- evoked efflux of excitatory amino acids in the guinea-pig cochlea in vitro. Exp. Brain Res. 121, 425-432.
Klinke, R., 1986. Neurotransmission in the inner ear. Hear. Res. 22, 235-243.
Klinke, R., Oertel, W., 1977. Amino acids - putative afferent transmitter in the cochlea? Exp. Brain Res. 30, 145-148.
Koch, R.A., Barish, M.E., 1994. Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. J. Neurosci. 14, 2585-2593.
Lipton, S.A., Rosenberg, P.A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. N. Engl. J. Med. 330, 613-622.
Matsubara, A., Laake, J.H., Davanger, S., Usami, S., Ottersen, O.P., 1996. Organization of AMPA receptor subunits at a glutamate synapse: A quantitative immunogold analysis of hair cell synapses in the rat organ of Corti. J. Neurosci. 16, 4457-4467.
Muir, K.W., Lees, K.R., 1995. Clinical experience with excitatory amino acid antagonist drugs. Stroke 26, 503-513.
Niedzielski, A.S., Wenthold, R.J., 1995. Expression of AMPA, kainate, and NMDA receptor subunits in cochlear and vestibular ganglia. J. Neurosci. 15, 2338-2353.
Oestreicher, E., Ehrenberger, K., Felix, D., 2002. Different action of memantine and caroverine on glutamatergic transmission in the mammalian cochlea. Adv. Otorhinolaryngol. 59, 18-25.
Ohinata, Y., Miller, JM., Schacht, J., 2003. Protection from noise-induced lipid peroxidation and hair cell loss in the cochlea. 966, 265-273.
Puel, J.L., 1995. Chemical synaptic transmission in the cochlea. Prog. Neurobiol. 47, 449-476.
Puel, J.L., Pujol, R., Tribillac, F., Ladrech, S., Eybalin, M., 1994. Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. J. Comp. Neurol. 341, 241-256.
Puel, J.L., Ruel, J., D'aldin, C.G., Pujol, R., 1998. Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. Neuroreport 9, 2109-2114.
Pujol, R., Lenoir, M., Robertson, D., Eybalin, M., Johnstone, B.M., 1985. Kainic acid selectively alters auditory dendrites connected with cochlear inner hair cells. Hear. Res. 18, 145-151.
Pujol, R., Puel, J.L., d'Aldin, C.G., Eybalin, M., 1992. Physiopathology of the glutamatergic synapses in the cochlea. Acta Otolaryngol. 113, 330-334.
Robertson, D., 1983. Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hear. Res. 9, 263-278.
Ryan, A.F., Brumm, D., Kraft, M., 1991. Occurrence and distribution of non-NMDA glutamate receptor mRNAs in the cochlea. Neuroreport 2, 643-646.
Saunders, J.C., Rhyne, R.L., 1970. Cochlear nucleus activity and threshold shift in cat. Brain Res. 24, 336-339.
Shero, M., Salvi, R.J., Chen, L., Hashino, E., 1998. Excitotoxic effect of kainic acid on chicken cochlear afferent neurons. Neurosci. Lett. 27, 81-84.

Spoendlin, H., 1971. Primary structural changes in the organ of corti after acoustic overstimulation. Acta Otolaryngol. (Stockh.) 71, 166-176.
Usami, S., Matsubara, A., Fujita, S., Shinkawa, H., Hayashi, M., 1995. NMDA (NMDAR1) and AMPA-type (GluR2/3) receptor subunits are expressed in the inner ear. Neuroreport 6, 1161-1164.
Zivin, J.A., Choi, D.W., 1991. Stroke therapy. Sci. Am. 265, 56-63.