An acute experimental model of hearing loss using noise in combination with hypoxia and its use in the investigation of a putative cochleoprotectant

Thesis submitted for the Degree of
Doctor of Philosophy
at the University of Leicester

by

Steven Etheridge BSc
Centre for the Mechanisms of Human Toxicology
University of Leicester

September 2002

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

ABSTRACT

An acute experimental model of hearing loss using noise in combination with hypoxia and its use in the investigation of a putative cochleoprotectant

Mr Steven Etheridge

The aim of this study was to develop a model of acute noise induced hearing loss that focused on excitotoxic damage, in the albino guinea pig. The model used moderate 12% hypoxia in combination with 100 dB SPL bandpass 5-20 kHz noise. The model employed gross electrophysiological measures as auditory endpoints, comprising the brainstem auditory evoked response (BAER) the cochlear compound action potential (CAP), and the ensemble spontaneous or driven cochlear nerve activity (ESAC/EDAC) derived from the Fast Fourier Transform of gross cochlear nerve (CN) activity.

Preliminary model development was carried out using BAER. These experiments established an optimal sedation and hypoxia regimen, demonstrating that systemic physiological stability was maintained with this regime. Significant changes in the BAER were seen within the first 15 minutes of exposure with noise and noise/hypoxia. There was significant synergy between noise and hypoxia, but hypoxia alone did not result in any significant changes in BAER.

More detailed development of the model utilised frequency specific CAP threshold, amplitude and latency measurements at 8, 16, 24 and 30 kHz. Significant frequency dependent changes were seen in all these parameters that again revealed synergy between acute noise and hypoxia. These results inferred excitotoxic damage at the afferent nerve synapse, and damage due to metabolic overload further downstream in the afferent nerve fibre. The ESAC/EDAC results also supported this.

The above model was then used to show that a single oral dose (20mg/kg) of lamotrigine (LTG) provided cochleoprotection against acute noise. LTG rapidly penetrated the inner ear and there was marginal evidence of a tonotopic effect on CAP threshold and amplitude, but no effect on ESAC. LTG also ameliorated noise induced threshold shifts at 8 and 16 kHz by approximately 10 dB. The EDAC signal during noise exposure was also significantly reduced. These results suggest that LTG acts by reducing driven activity of the CN and thus reduces metabolic overload on the nerve through its voltage dependent action on the Na^+ channel.

DECLARATION

This thesis is based on work conducted by the Author in conjunction with the Centre for the Mechanisms of Human Toxicology, MRC Toxicology Unit, Leicester University and GlaxoSmithKline Pharmaceuticals (GSK) during the period September 1995 and September 2001.

Experimental work over the period 1st April 1999 to 30th April 2000, carried out at GSK Stevenage, was performed in collaboration with Mr David Selvadurai, Leicester Royal Infirmary, Leicester.

The work in this thesis is original, unless otherwise acknowledged in the text or by references.

ACKNOWLEDGEMENTS

I would especially like to thank the following people who have been instrumental in the making of this thesis. Firstly, my supervisor and friend Dr Mike Mulheran for his commitment, scientific knowledge, enthusiasm and disgraceful, but highly amusing sense of humour.

To Dr Patrick Jones for helping me in the build and design of a laboratory and data collection system that were pivotal in the success of the work presented here.

First class surgical expertise, and a mate to share the burdens of the laboratory were provided by Dr. David Selvadurai, whilst Lisa Ranshaw provided expert assistance in the pharmacokinetic analysis of lamotrigine.

Dr. Steve Damment and Dr. David Tweats for providing continued support during difficult times, and all the skilled technicians that have assisted me during the course of this work, especially those that provided expert care to the guinea pigs.

Thanks also to Steve Papworth and Dr David Furness for furnishing me with the necessary skills enabling me to develop cochlear histopathology techniques – even though I couldn't include the work in this thesis.

Lastly, I would like to thank the most important people in my life; my wife, Helen, and the 'crazy gang' Samuel and Harry for putting up with me during the long dark periods of this thesis.

GLOSSARY OF ABBREVIATIONS

ABR	Auditory brainstem response
ACh	Acetylcholine
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ARP	Absolute refractory period
ATP	Adenosine triphosphate
BAER	Brainstem auditory evoked response
BM	Basilar membrane
BPM	Beats per minute
Ca^{2+}	Calcium
CF	Characteristic frequency
CAP	Compound action potential
CF	Characteristic frequency
Cl	Chloride
CM	Cochlear microphonic potential
CN	Cochlear nerve
CO	Carbon monoxide
dB	Decibel
ECG	Electrocardiography
EPSP	Excitatory post synaptic potential
ESAC	Ensemble spontaneous cochlear nerve activity
EDAC	Ensemble driven cochlear nerve activity
EP	Endocochlear potential
EPSP	Excitatory post synaptic potential
FFT	Fast fourier transformation
GABA	Gamma-aminobutyric acid
GSK	GlaxoSmithKline
K^+	Potassium
KA	Kainate
LTG	Lamotrigine
M	Molar
mM	Millimole
ms	Millisecond
Na^+	Sodium
NIHL	Noise induced hearing loss
NMDA	N-Methyl-D-Aspartate
OAE	Oto-acoustic emissions
PTS	Permanent threshold shift
SG	Spiral ganglion
SMA	Spiral modiolar artery
SMV	Spiral modiolar vein
SP	Summating potential
SPL	Sound pressure level
SR	Spontaneous rate
TTS	Temporary threshold shift
µV	Microvolt
µM	Micromole
VGSC	Voltage gated sodium channel
VDSCM	Voltage dependent sodium channel modulator
VGCC	Voltage gated calcium channel
VDCCM	Voltage dependent calcium channel modulator

Chapter 1. General introduction and rationale for the study

There is a broad spectrum of hearing deficit in the world population due to a number of clearly identified causes. These include; noise exposure, ototoxic damage, loss due to viral and bacterial infection eg meningitis, and the long term loss due to ageing (Chiodo *et al* 1994). As a target organ for potential therapy to treat this sensory deficit, the inner ear or cochlea has been largely neglected. This is unfortunate, as there is a very significant unmet need in terms of how frequently hearing deficit presents as a health problem (internal GSK communication, Davis 1983, Chiodo *et al* 1994).

Whilst there has been little progress in addressing this clinical need, there has been more progress made in understanding the basic mechanisms underlying hearing loss, over the last four decades or so. This body of work first focused on the physical correlates of cochlear damage, with interest in the biochemical mechanisms following later (Henderson *et al*, 1995, 1999). This earlier work was followed by the discovery that glutamate was the afferent neurotransmitter in the cochlea (Pujol *et al* 1990, Puel *et al* 1991). This led to characterising the contribution made by excitotoxic damage to the cochlear nerve (CN) following ototrauma such as noise (Pujol *et al*, 1992, 1993, 1994, 1995, 1998). A component of hearing loss due to excitotoxicity was also proposed to be a common feature to other ototraumas apart from noise. It was also suggested to contribute to age related hearing loss and some forms of tinnitus (Pujol *et al* 1990, 1993, Puel *et al* 1999).

The role of excitotoxic damage in hearing deficit served as the original stimulus to the work presented in this thesis. The particular aim was to develop an acute animal model of excitotoxicity that maximised the potential contribution to hearing deficit caused by excitotoxicity. After consideration of the literature, it was decided to develop this model using moderate noise levels to reduce the involvement of physical damage in the cochlea (Mills *et al* 1979, Miller 1984, Hamernick *et al* 1986, Puel *et al* 1988).

To accelerate the effect of any excitotoxic component, it was also proposed to use, in combination with noise, a moderate level of hypoxia that would not compromise systemic physiological function (Kiedrowski *et al* 1994a-b, Taylor and Meldrum 1995). If systemic function were affected, this would have a further confounding effect on auditory function independent of any excitotoxicity (Evans 1972, 1974, Mulheran 1990).

Therefore, it was important to establish what level of hypoxia would lead to a disturbance in glutamate re-uptake, whilst leaving cardiorespiratory function unaffected. This had been investigated previously to some extent, but using a rat model with co-exposure to carbon monoxide instead of marginal atmospheric oxygen (Young *et al* 1987, Fechter *et al* 1988).

Once an acute model of cochlear excitotoxicity was developed, it was then intended to employ this model to investigate agents that would possibly protect the cochlea against excitotoxic damage. Some success with the experimental glutamate antagonists such as MK-801, D-AP5 and DNQX, had already been reported by other workers (Puel *et al* 1994, Chen *et al* 1999). However, the aim here was to use GSK compounds that had already been approved for clinical use, thereby realising the possibility of therapeutic use.

Overall, the following hypotheses were proposed for this study:

1. Moderate, acute noise insult with or without hypoxia, for a short period of time, will result in changes to excitatory neurotransmission in the cochlear nerve (CN) as assessed by electrophysiological measurements.

2. A combination of noise and hypoxia (NH) will result in synergistic effects due to increased metabolic demand on the CN.

3. A proportion of the observed electrophysiological deficit caused by exposure to modest acute noise or NH exposure is due to the modulation of excitatory neurotransmission. The resultant outcome is increased glutamate levels at the level of the afferent nerve synapse resulting in excitotoxicity.

4. The measurement of spontaneous CN activity offers an insight into 'real time' activity and as such is a good measure of both CN metabolic demand and ion channel activity.

5. Pharmacological agents that modulate excitatory neurotransmission play a role in the protection of the cochlear against noise or NH induced hearing loss.

6. Based on the above, it was believed that lamotrigine, a voltage dependent sodium channel modulator (VDSCM) could offer pharmacological protection against NIHL.

1.2. The anatomy of the middle and inner ear

The relevant anatomy of the ear is reviewed in the following sections.

1.2.1. The middle ear

After being channelled by the external ear, the sound energy is delivered to the cochlea by the middle ear. The function of the middle ear is to match the air-fluid impedance by collecting sound energy over the larger surface area of the tympanic membrane, and delivering this to the much smaller surface area presented by the oval window. Transmission is achieved by three small bones known as the ossicles. These are; the maleus which is attached to the tympanic membrane, the incus and the stapes (Pickles, 1991).

The stapes is attached to the flexible oval window (figure 1.2) in the wall of the cochlea allowing the transmission of sound. At high stimulus intensities (greater than 100dB SPL) the middle ear muscles can attenuate transmission of frequencies up to about 2 kHz. Contraction of these muscles increases stiffness of the ossicular chain and consequently reduces transmission of the lower frequencies (Pickles, 1991).

1.2.2. The inner ear

1.2.2.1. The cochlea

The mammalian cochlea is normally embedded deep in the temporal bone of the skull. Unusually, in the guinea pig the cochlea is remarkably superficial and is contained effectively within the middle ear cavity, making it especially suitable for experimental work. The cochlea itself has a snail-like, helical appearance typically of 3.5 to 4 turns in total with three distinct chambers; the scala tympani, the scala vestibuli and the scala media (figures 1.1 and 1.2). The scala vestibuli (separated from the stapes by the oval window) and tympani are joined at the apex by an opening known as the helicotrema, and are bathed in a fluid known as perilymph. This is similar to extracellular fluid with respect to its ionic composition. It has a high Na^+ concentration of about 150mM, a low K^+ concentration of about 3 to 4mM and a Ca^{2+} concentration of about 2mM. The pH of perilymph is approximately 7.88 (Miller, 1985).

The scala media lies in between the two outer compartments and is enclosed by the stria vascularis, Reissner's membrane and the basilar membrane (BM). It is also fluid filled containing endolymph, and is unique in that it has a high intracellular K^+ concentration of about 144mM and low Na^+ concentration of about 9mM. Endolymph has a pH ranging from 7.44 to 7.82 (Miller, 1985) and due to the high K^+ concentration it also has a high resting potential of +80mV.

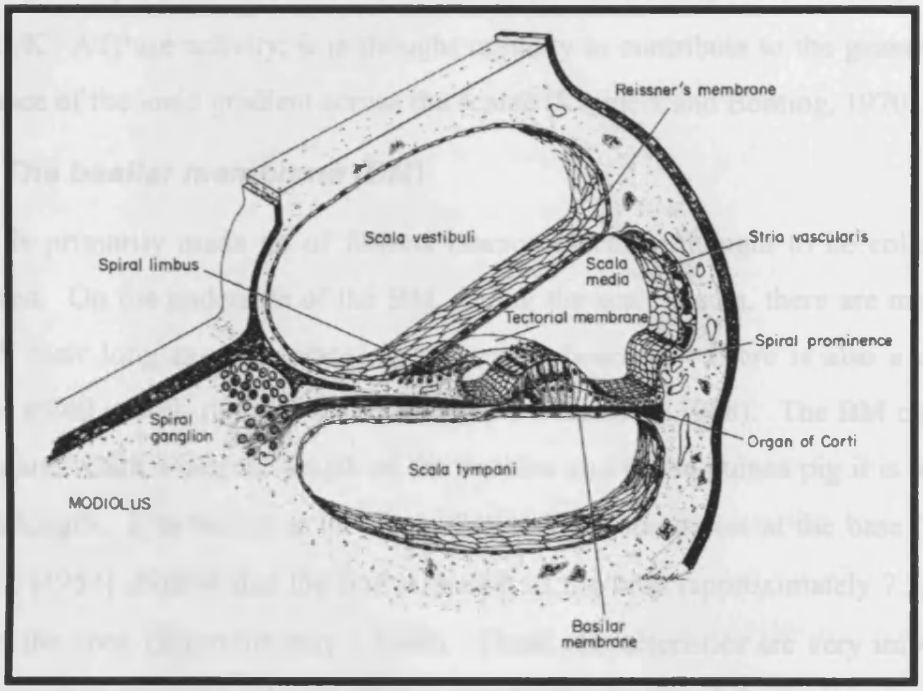


Figure 1.1 Cross-section of the cochlea showing the three fluid filled scalae, the organ of Corti and the stria vascularis. Taken from Pickles, 1991.

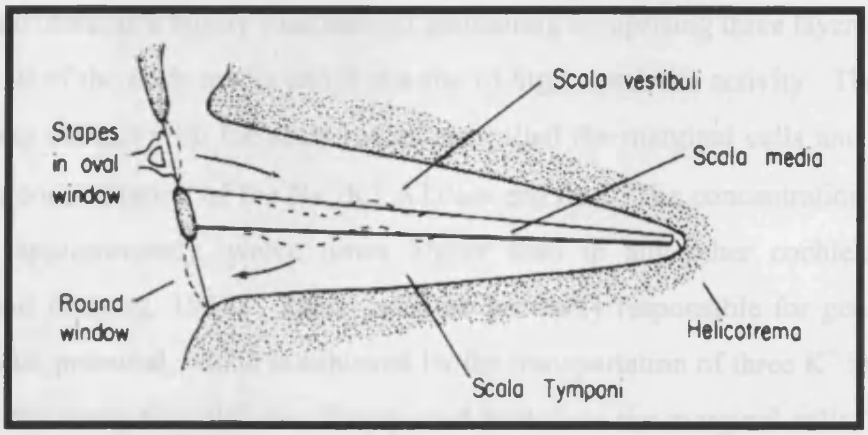


Figure 1.2. The cochlea – uncoiled. Taken from Pickles, 1991.

The chamber containing scala media is known as the cochlear duct and comprises the following important structures:

1.2.2.2. Reissner's membrane

This is a thin membrane made up of two cell layers (about 10-15μM thick) and it separates the scala vestibuli from the scala media (along with the BM and the stria vascularis) to form the fluid filled compartment known as the cochlear duct (figure 1.2). The side adjacent to the scala media shows some specialisation, with tight cell junctions to restrict the passage of large molecules. The cells in this layer have a large surface area with a large complement of microvilli projecting into the scala media and it does appear that this layer is permeable to both K^+ and Na^+ (Mnich, 1971). Whilst it does exhibit some Na^+/K^+ ATPase activity, it is thought unlikely to contribute to the generation and maintenance of the ionic gradient across the scalae (Kuijpers and Bonting, 1970).

1.2.2.3. The basilar membrane (BM)

The BM is primarily made up of fibrous macromolecules, thought to be collagen and fibronectins. On the underside of the BM, facing the scala media, there are mesothelial cells with their long axes orientated in the spiral direction. There is also a capillary, called the spiral vessel, running through the BM (Axelsson, 1968). The BM changes in thickness and width along the length of the cochlea and in the guinea pig it is about 18-20mm in length. It is widest at the apex (250μm) and narrowest at the base (150μm). Fernandez (1952) showed that the BM is thicker at the base (approximately 7.5μM) and thinner at the apex (approximately 1.4μM). These characteristics are very important in cochlear function are will be dealt with in more detail in section 1.3.

1.2.2.4. The stria vascularis

The stria vascularis is a highly vascularised epithelium comprising three layers located on the outer wall of the scala media and it is a site of high metabolic activity. The epithelial cells in direct contact with the scala media are called the marginal cells and these cells have a high concentration of the Na^+/K^+ ATPase enzyme. The concentration of Na^+/K^+ ATPase is approximately twelve times higher than in any other cochlear structure (Kuijpers and Bonting, 1969). These cells are primarily responsible for generating the endolymphatic potential, which is achieved by the transportation of three K^+ ions into the endolymph for every two Na^+ ions transported back into the marginal cells. The stria, being the most metabolically active tissue in the cochlea, has the greatest utilisation of blood glucose both at rest and during stimulation (Ryan, 1988).

1.2.2.5. The spiral limbus and tectorial membrane

The spiral limbus is a vascularised connective tissue supported by the spiral lamina, the shelf of bone that projects into the cochlea (figure 1.1). Its function is to include the support, production and maintenance of the components of the tectorial membrane (Santi, 1988).

The tectorial membrane is fixed, only on its inner edge, where it attaches to the spiral limbus although it is joined to the reticular laminar by small processes (Pickles, 1991). The tectorial membrane contains two distinct areas of specialisation; Hensen's stripe and Hardesty's membrane. The hairs of the inner hair cells fit loosely into the groove known as Hensen's stripe on the underside of the tectorial membrane and the tallest stereocilia on the outer hair cells appear to be embedded (not deeply) into the Hardesty's membrane. The biomechanical properties of the tectorial membrane are also considered to be of importance in determining both the sensitivity and frequency selectivity of the cochlea.

1.2.2.6. The organ of Corti

The organ of Corti is situated on the BM and contains one row of inner/internal hair cells (IHC) and 3 or 4 rows of outer/external hair cells (OHC). These sensory hair cells fulfil very different physiological roles. The OHCs act as stimulus intensity dependent amplifiers and attenuators. Their principle role is to enhance sensitivity and selectivity of the cochlea. The IHCs serve as the sensory transducers proper and convert movement of the BM into neural signal (figure 1.3). Also present is an arch of rods or pillar cells, which gives the organ of Corti its rigidity. The upper end of the rods ends in the reticular lamina that forms the true chemical division between the ions in the fluids of the scala media and the scala tympani. Phalangeal cells or Deiters' cells are also present which serve to support and precisely orientate the IHCs and OHCs. Supporting cells are involved in the regulation of the ionic microenvironment and reuptake of neurotransmitter (Santi, 1988).

1.2.2.7. The sensory cells

The sensory cells are termed hair cells because of the specialised projections on their apical surface termed stereocilia. The descriptors inner and outer, refer to their relative positions to the central modiolar axis of the cochlea.

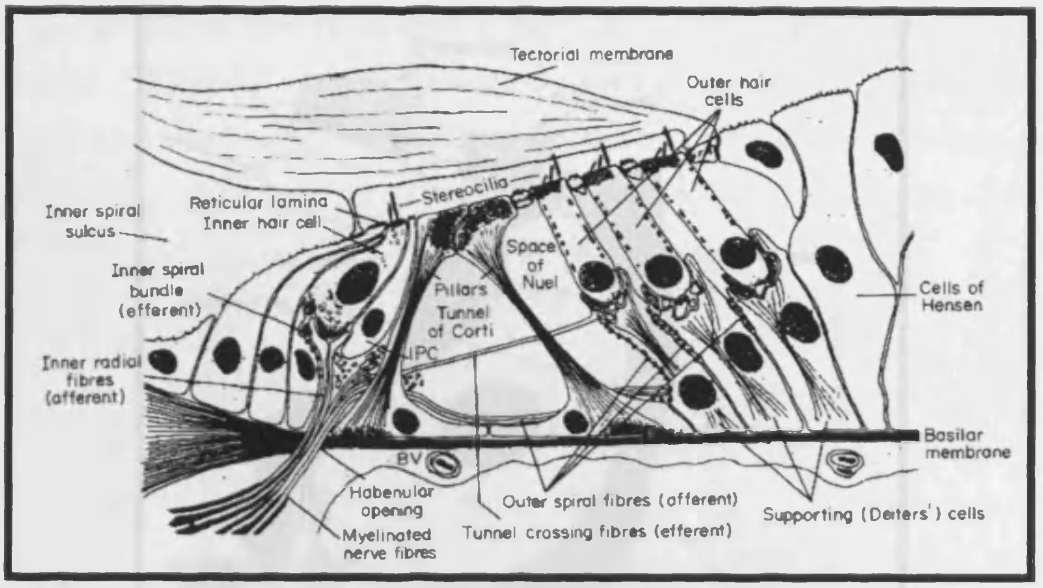


Figure 1.3. Cross section of the organ of Corti showing the precise geometric relationship of the various cell types (taken from Pickles, 1991).

1.2.2.8. Inner Hair Cells (IHCs)

The IHCs comprise a single row of 'flask shaped' cells from the base to the apex of the cochlea. They are about 35μm long and contain a central nucleus surrounded by numerous mitochondria as shown in figure 1.4 (Furness *et al*, 1990). Smooth endoplasmic reticulum becomes further specialised as a cellular organelle to form the subsurface cisternae which is contiguous with the cell membrane (Lim, 1986).

The vast majority of cochlear afferent fibres originate from the IHCs, reflecting their principle role in the transduction of acoustic stimuli into neuronal signals.

The primary neurotransmitter at the IHC synapse is glutamate (figure 1.5, Pujol, 1994). On the presynaptic membrane of IHCs, the synapse is often marked by small dense synaptic bars or invaginations at right angles to the cell wall, or by rounded synaptic bodies together with synaptic vesicles (Ades and Engstrôm, 1976).

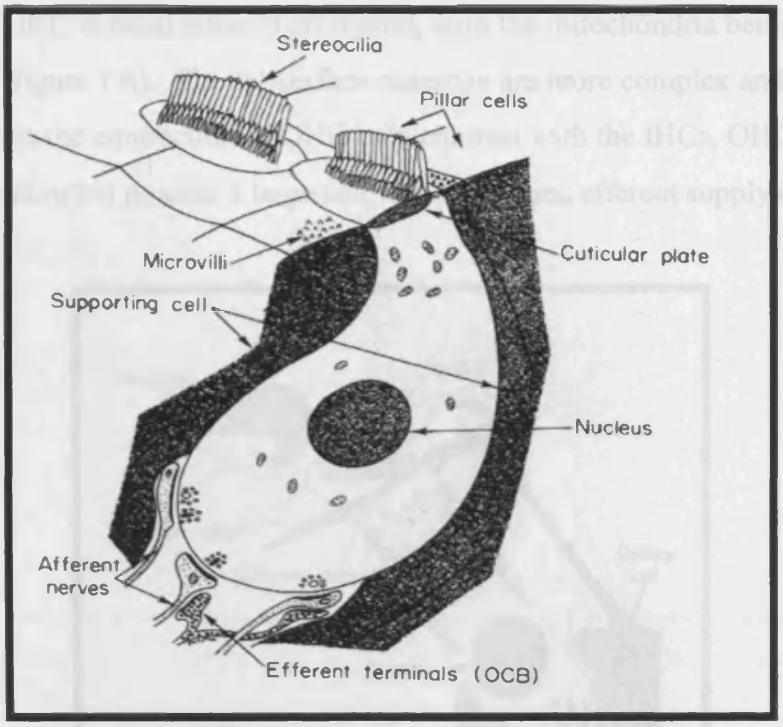


Figure 1.4. An illustration of the inner hair cell (IHC) showing afferent and efferent innervation and arrangement of apical stereocilia. Taken from Furness *et al*, 1990.

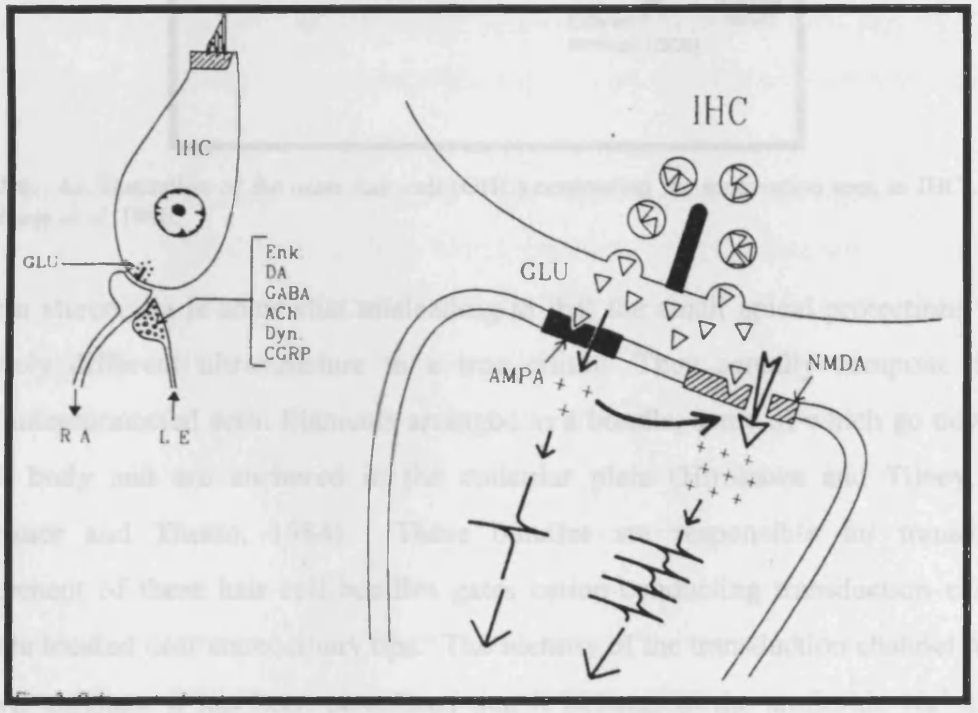


Figure 1.5. A schematic diagram of the IHC afferent synapse, highlighting the importance of the neurotransmitter glutamate (GLU). Taken from Pujol, 1994.

1.2.2.9. Outer hair cells (OHCs)

There are typically three rows of outer hair cells (OHCs) and their morphology is cylindrical and elongated. The length of these cells increases from the base to the apex of the cochlea from about 30 to 70μm in the guinea pig (Zajic and Schacht, 1987). The

nucleus of the OHC is basal rather than central, with the mitochondria being below rather than around it (figure 1.6). The subsurface cisternae are more complex and multi-layered and play a role in the contraction of OHCs. In contrast with the IHCs, OHCs have a poor afferent innervation but possess a large and well developed efferent supply (figure 1.7).

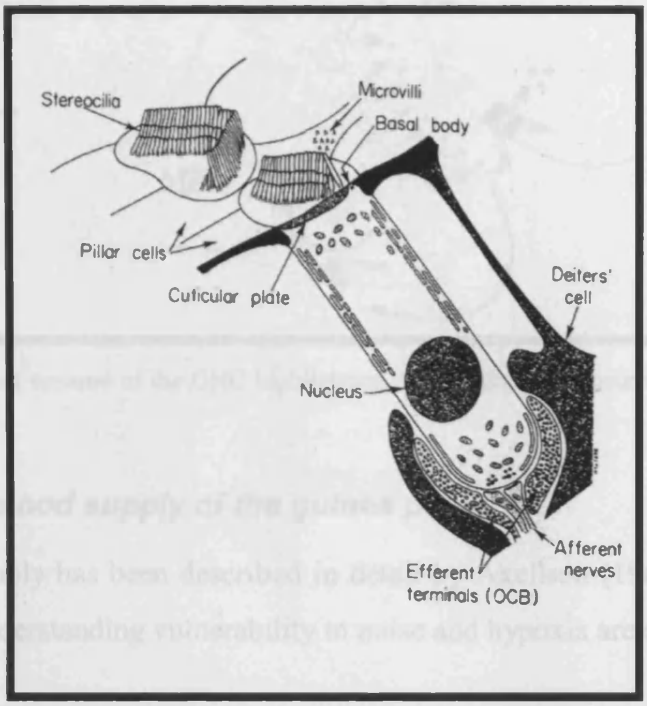


Figure 1.6. An illustration of the outer hair cell (OHC) contrasting the innervation seen in IHCs. Taken from Furness *et al*, 1990.

The term stereocilia is somewhat misleading in that the small apical projections have a completely different ultrastructure to a true cilium. They actually comprise densely packed interconnected actin filaments arranged in a bundle, some of which go down into the cell body and are anchored in the cuticular plate (Hirokawa and Tilney, 1982, Neugebauer and Thurm, 1984). These bundles are responsible for transduction. Displacement of these hair cell bundles gates cation-conducting transduction channels, which are located near stereociliary tips. The identity of the transduction channel remains unknown although it has been postulated that it belongs to the amiloride Na^+ channel family (Gillespie, 1995).

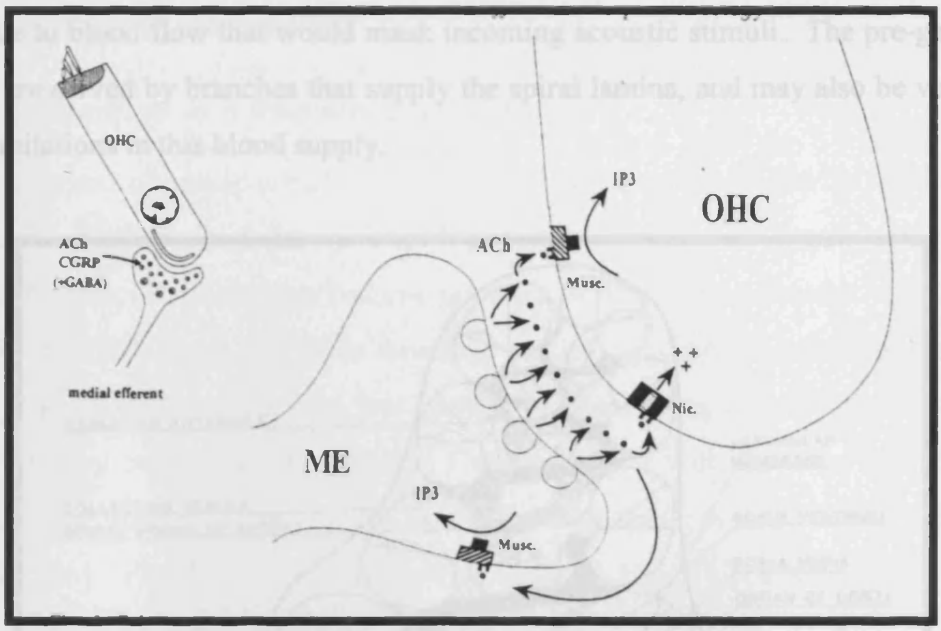


Figure 1.7. The efferent synapse of the OHC highlighting ACh as the major neurotransmitter. Taken from Pujol, 1994.

1.2.3. Cochlear blood supply of the guinea pig

Cochlear blood supply has been described in detail by Axellson (1968) and the features most relevant to understanding vulnerability to noise and hypoxia are discussed below.

The anatomy of the cochlear blood supply is schematically shown in figures 1.8 and 1.9. The inner ear is supplied by one main blood vessel namely the internal auditory artery which gives rise to the spiral modiolar artery (SMA). The SMA gives rise to many radiating arterioles that supply the cochlea and CN as shown in figure 1.9. In terms of functional supply, the stria vascularis is the best served tissue in the cochlea with a rich vascular 'net'. This matches the high demand for substrate required by the Na^+/K^+ ATPase pumps.

The larger body of myelinated CN fibres within the modiolus have their own network of radiating arterioles and capillary supply. In contrast the sensory epithelium of the organ of Corti does not have a direct blood supply. The nearest supply is to the spiral limbus. There is a single capillary running through the BM underneath the tunnel of Corti. The walls of the scala tympani and vestibuli are not particularly well vascularised. This means that both OHCs and IHCs are dependent on passive diffusion of oxygen from vessels which are situated up to 30 microns away, which infers that they may be particularly vulnerable at times of substantial metabolic demand (Nuttall and Lawrence, 1980). The reason for this poor vascularisation of the organ of Corti is most likely to reduce any noise

signal due to blood flow that would mask incoming acoustic stimuli. The pre-ganglionic neurites are served by branches that supply the spiral lamina, and may also be vulnerable due to limitations in this blood supply.

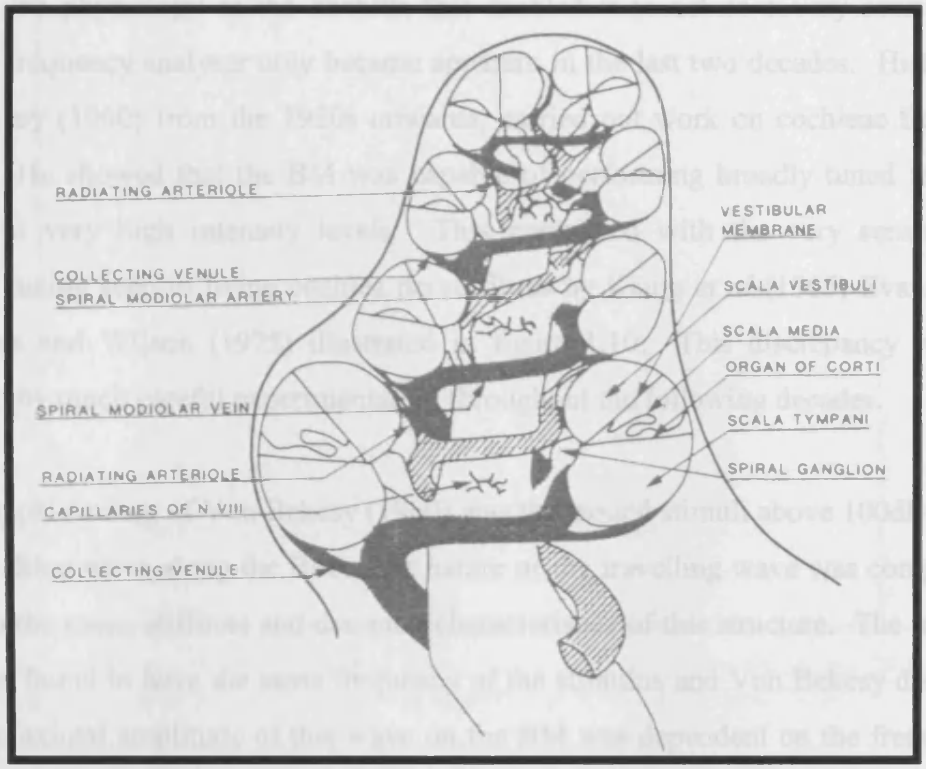


Figure 1.8. Guinea pig cochlea, radial section showing the SMA and SMV (reproduced from Axelsson, 1968).

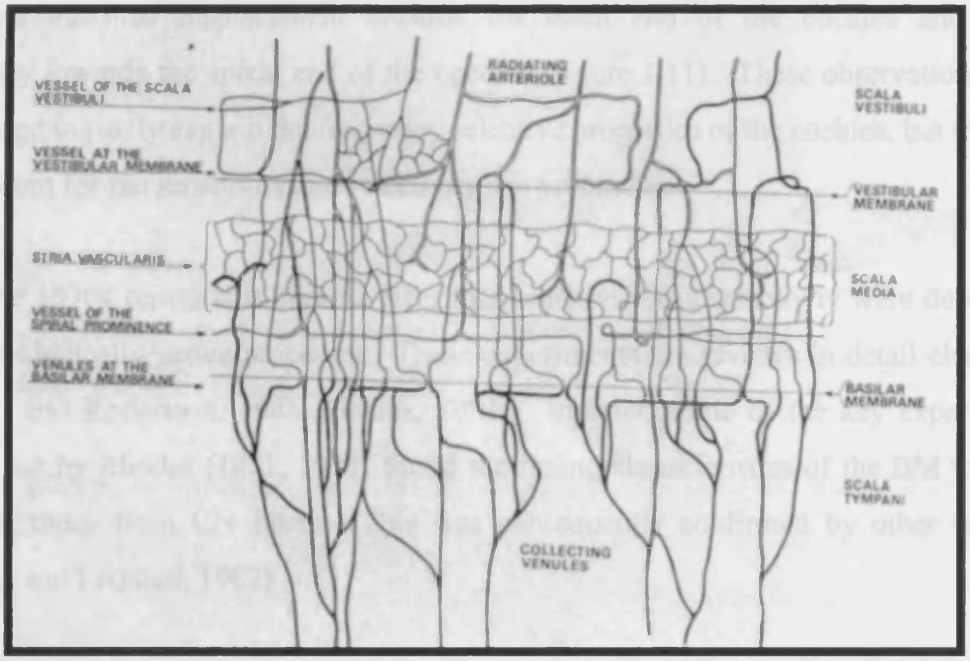


Figure 1.9. Schematic representation of the external wall of the cochlea (radial section) in the guinea pig and man (reproduced from Axelsson, 1968).

1.3. Physiology of the cochlea

1.3.1. The cochlea as a frequency analyser

The anatomy and ultrastructure of the cochlea had been well described by the 1960s. In contrast, the physiology of the cochlea that enabled it to act as a very sensitive and selective frequency analyser only became apparent in the last two decades. Historically, Von Bekesy (1960) from the 1930s onwards, carried out work on cochleae from dead animals. He showed that the BM was capable of performing broadly tuned frequency analysis at very high intensity levels. This contrasted with the very sensitive and selective tuning seen in living cochlea nerve fibres by Kiang *et al* (1965) Evans (1972) and Evans and Wilson (1975) illustrated in figure 1.10. This discrepancy was only explained by much careful experimentation throughout the following decades.

The principal finding of Von Bekesy (1960) was that sound stimuli above 100dB SPL set up a travelling wave along the BM. The nature of the travelling wave was complex and related to the mass, stiffness and damping characteristics of this structure. The travelling wave was found to have the same frequency of the stimulus and Von Bekesy discovered that the maximal amplitude of this wave on the BM was dependent on the frequency of the stimulus. After reaching its maximal amplitude the wave would die away. This tonotopic behaviour of the BM was arranged in such a way that high frequency stimuli produced maximal displacement towards the basal end of the cochlea and lower frequency towards the apical end of the cochlea (figure 1.11). These observations were considered to partly explain the frequency selective properties of the cochlea, but they did not account for the sensitivity and selectivity shown *in vivo*.

From the 1970s onwards it became clear that sensitivity and selectivity were dependent on physiologically active processes. These experiments are reviews in detail elsewhere (Patuzzi and Robertson, 1988, Pickles, 1991). In brief, some of the key experiments carried out by Rhodes (1971, 1978) found the tuning characteristics of the BM to be as sharp as those from CN fibres. This was subsequently confirmed by other workers (Khanna and Leonard, 1982).

Other workers also performed electrophysiological studies on IHCs and OHCs *in vivo,* showing the sensitivity and selectivity exhibited in both CN fibres and the BM (Russell and Sellick, 1978).

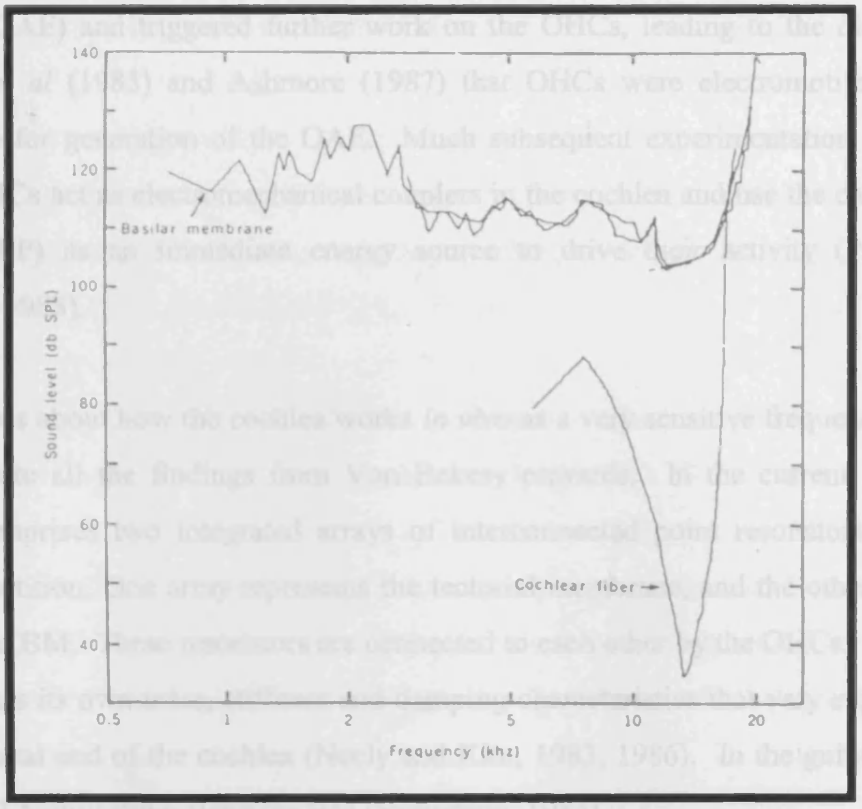


Figure 1.10. Comparison of BM and neural tuning curves as performed by Evans and Wilson (1975), showing the large discrepancies between the two (Adapted from Evans and Wilson, 1975).

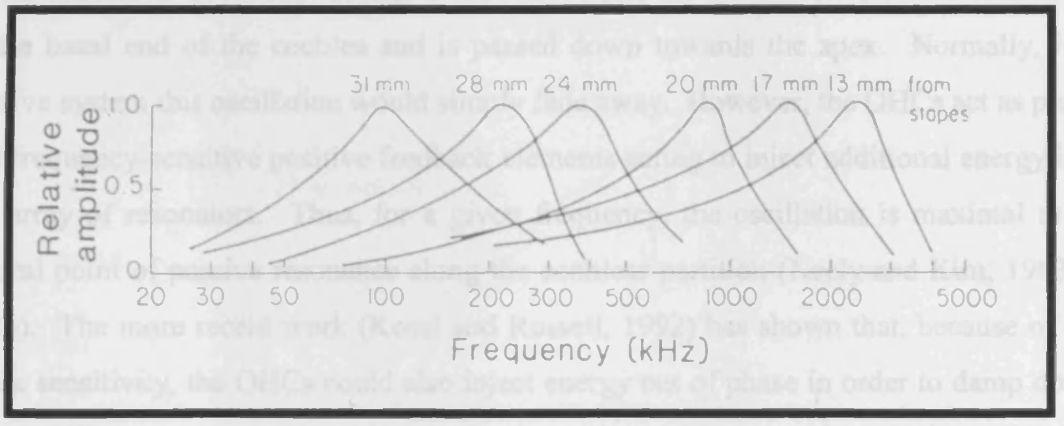


Figure 1.11. The frequency response of the BM at six different points along the cochlear partition. The position where the measurement was made with respect to the stapes is given with each curve. Adapted from Pickles (1982).

A large step in understanding the active process underlying cochlear sensitivity and selectivity was made by Kemp (1978) who applied discrete, acoustic stimuli into the sealed auditory meatus in humans. This produced a delayed acoustic response, of which the timing and amplitude characteristics could not be explained by passive echo. From this Kemp proposed that there was an 'active' transduction process in the cochlea that produced a coherent release of mechanical energy that was transmitted back through the

BM, the middle ear chain and the tympanic membrane. This was termed the *otoacoustic emission* (OAE) and triggered further work on the OHCs, leading to the discovery by Brownell *et al* (1985) and Ashmore (1987) that OHCs were electromotile and were responsible for generation of the OAE. Much subsequent experimentation has shown that the OHCs act as electromechanical couplers in the cochlea and use the endocochlear potential (EP) as an immediate energy source to drive their activity (Patuzzi and Robertson, 1988).

Current ideas about how the cochlea works *in vivo* as a very sensitive frequency analyser now integrate all the findings from Von Bekesy onwards. In the current model, the cochlea comprises two integrated arrays of interconnected point resonators along the cochlear partition. One array represents the tectorial membrane, and the other the organ of Corti and BM. These resonators are connected to each other by the OHCs. Each point resonator has its own mass, stiffness and damping characteristics that vary exponentially from the basal end of the cochlea (Neely and Kim, 1983, 1986). In the guinea pig, this equates to 2.5mm/octave along the BM (Robertson, 1984).

In this model, when acoustic energy is introduced into the system, the oscillation starts up at the basal end of the cochlea and is passed down towards the apex. Normally, in a passive system this oscillation would simply fade away. However, the OHCs act as phase and frequency-sensitive positive feedback elements acting to inject additional energy into the array of resonators. Thus, for a given frequency, the oscillation is maximal at its natural point of passive resonance along the cochlear partition (Neely and Kim, 1983 & 1986). The more recent work (Kossl and Russell, 1992) has shown that, because of the phase sensitivity, the OHCs could also inject energy out of phase in order to damp down the travelling wave as it proceeds apically down the cochlear partition. This mechanism also appears to operate at higher intensities to damp down excessive motion, providing some protection to the nerve fibres against excessive discharge rates.

In summary, all this information identified by the aforementioned experimental and theoretical workers combines the following features:

i) An *active amplification* process – This involved the OHCs picking up very low level signal ie. about 0 dB SPL or 10^{-17} W, and augmenting it by injecting mechanical energy

in phase with the stimulus up to levels of about 40-70 dB SPL. This, in turn, drives the depolarisation of the IHCs and in turn the CN.

ii) An *active attenuating* process – This involved the OHCs at higher stimulus intensities above 60 dB SPL or so, beginning to *actively attenuate* movement of the cochlear partition by and augmenting it by injecting mechanical energy *out of phase* with the stimulus up to levels of about 120 dB SPL.

iii) The *passive* process - This is performed to damp down the passive and steeper growth function of the BM in order to extend the dynamic range of the CN.

If (i) is damaged, this leads to higher thresholds and lower amplitudes. If both (i) and (ii) are damaged, this also leads to higher thresholds and lower amplitudes up to a given stimulus intensity, but then the unmasked passive growth function reveals itself above these levels. This is summarised in figure 1.12.

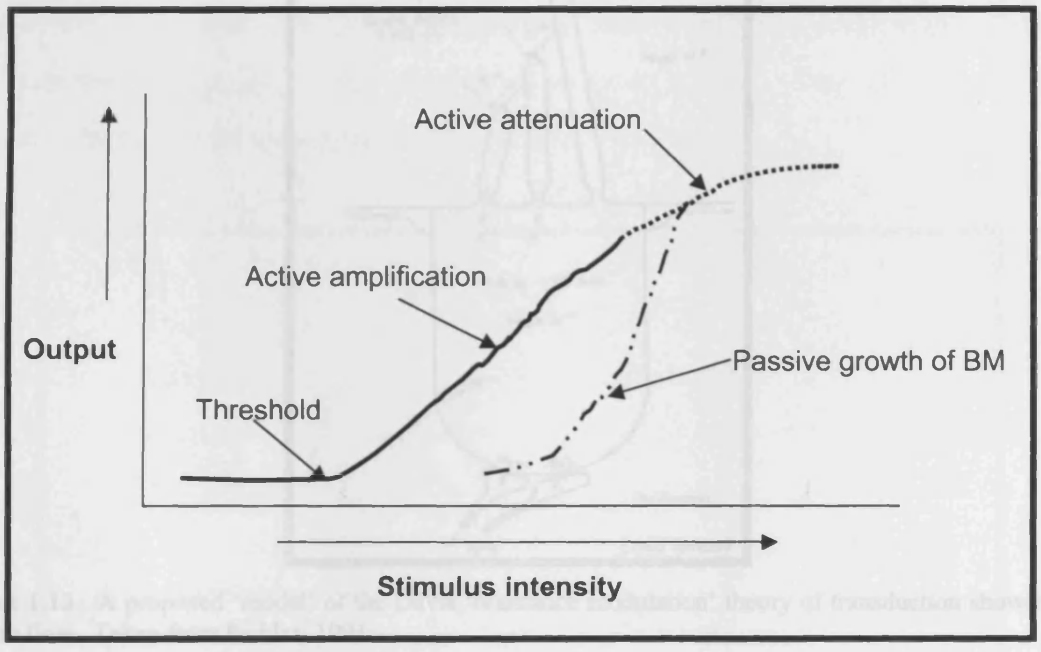


Figure 1.12. A schematic diagram showing the relationship between growth, active amplification and attenuation, and the passive growth function of the BM.

1.3.2. Transduction in OHCs and IHCs

The transduction roles played by OHCs and IHCs is different, but in both cases it is dependent on displacement of the stereocilia resulting from radial motion of the tectorial membrane as it moves with respect to the BM. In both cell types, depolarising current is

generated when the stereocilia are displaced towards their tallest row. Conversely, hyperpolarising (negative) receptor potentials are generated when the stereocilia are displaced in the opposite direction. Therefore, cell membrane potentials are controlled by hair cell stereocilia displacement (Hudspeth, 1985).

This deflection of the stereocilia causes an increased probability of the opening of transducer channels in the hair cell apical membrane. This then causes variation in the amount of current carried by K^+ through the hair cells. The drive for the K^+ current comes from the combination of the positive EP and the resting membrane potential of the hair cells, resulting in a potential difference of about 125mV. In OHCs, there are Ca^{2+}-activated K^+ channels that open allowing K^+ into the perilymph thus completing the transduction process (Kros and Crawford, 1988). In IHCs there are voltage activated K^+ channels that serve the same purpose (Kros and Crawford, 1988). Potassium is finally removed from the perilymph by the supporting cells (see figure 1.13).

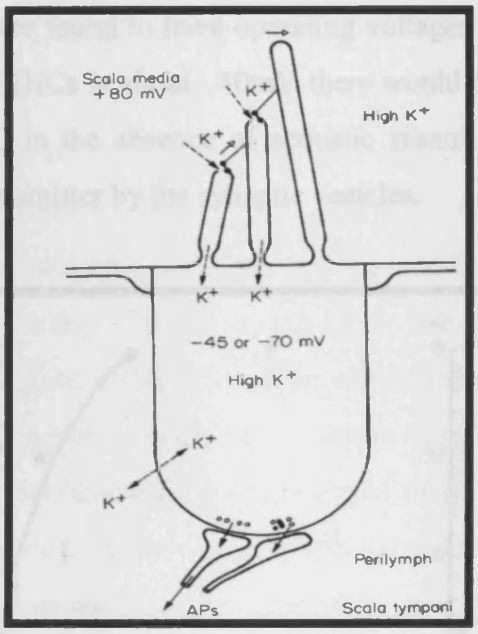


Figure 1.13. A proposed 'model' of the Davis 'resistance modulation' theory of transduction showing K^+ current flow. Taken from Pickles, 1991.

The resting potential of OHCs are typically about –75mV (Russell, 1987). In response to stimulation the OHCs produce both ac and dc currents, but it is the ac current that is physiologically important in the generation of electromotile activity. At rest there is always a standing K^+ current through the OHCs. When acoustic energy causes deflection of the stereocilia as described above, this results in variation of the current at the same frequency as the stimulus. This alternating current affects the conformation of proteins

within the cell membrane such that they contract and elongate to depolarising and hyperpolarising current respectively (Holley and Ashmore, 1988). These proteins are joined as filaments to produce a spring-like structure throughout the cell membrane of the OHC. When coupled to the tectorial and BM this causes an amplification of the passive movements of the cochlear partition resulting in an increase of up to at least x 1,000 (Hudspeth, 1985).

At rest the IHC membrane potential is about –40mV (Russell, 1987). In contrast with the OHCs it is the dc component of the receptor potential that is important for the transduction of acoustic stimuli into neural signal. The dc component arises from the combined properties of the transfer function of the stereociliary apical transduction channel and the capacitance properties of the IHCs (figure 1.14). This asymmetric transfer function leads to a net depolarisation during stimulation so as stimulus intensity is increased depolarisation is proportional. This depolarisation leads to an opening of voltage activated Ca^{2+} channels in the IHC basolateral membrane (Crawford & Kros, 1988). These channels were found to have operating voltages between –60 and –20mV. As the resting potential of IHCs is about –40mV there would be a considerable standing Ca^{2+} current into the IHC in the absence of acoustic stimuli. This Ca^{2+} current then regulates the release of transmitter by the synaptic vesicles.

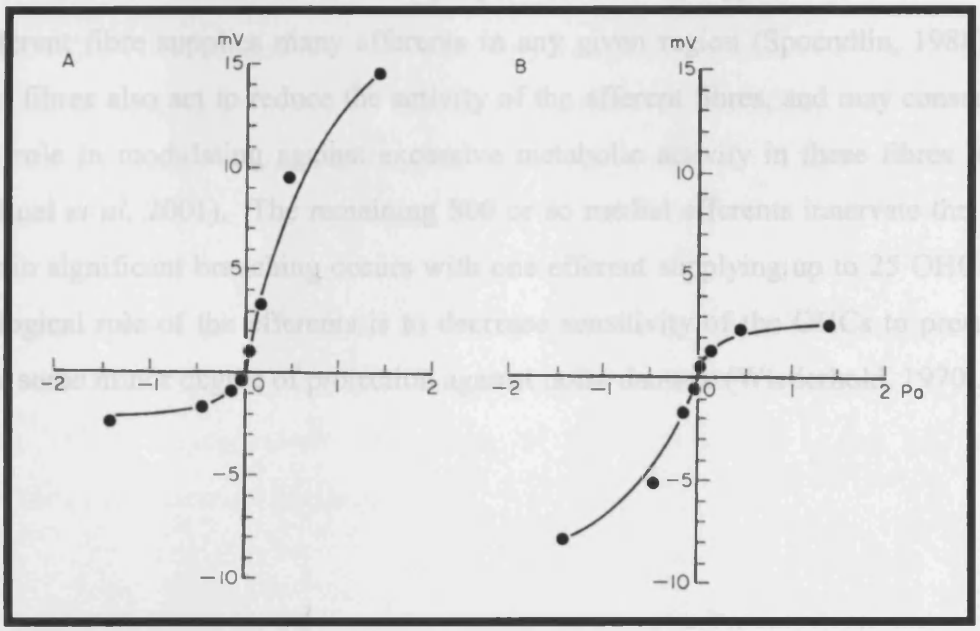


Figure 1.14. Input-Output functions of A) IHCs and B) OHCs. Plots represent intracellular voltage change (mV) against the stimulus pressure during sinusoidal stimulation (Pa). The depolarizing, upward current change for IHCs is clearly greater than the downward hyperpolarising change. The opposite is observed for OHCs. This only applies to low frequency sound stimuli where ac capacitance has not been attenuated by the capacitance of the cell wall. Adapted from Cody and Russell, 1987.

1.4. The cochlear nerve (CN)

The CN has been reviewed in detail elsewhere (Kiang *et al*, 1965, Evans 1975, Liberman and Kiang, 1978 and Pickles 1988). The majority of work characterising the fundamental anatomy and physiology of the nerve was done from the 1960s – 1980s and the main relevant features are summarised below.

The sensory innervation of the cochlea consists of an afferent and efferent supply (Morrison, 1975). There are about 40,000 afferent fibres in the guinea pig CN, classified as Type I and Type II fibres (Spoendlin, 1967) and shown in figures 1.15 and 1.16. Type I fibres constitute 85 to 90% of the afferent fibre population and each IHC has up to 20 synapses (Morrison, 1975). These fibres are unmyelinated for about 50-100 μM until they pass through the habenula perforata (figure 1.3) after which they become myelinated.

Type II fibres arise from the OHCs and are usually unmyelinated (Morrison, 1975). The role of these OHC afferent fibres is subject to speculation as no physiological activity has been recorded from them (Robertson, 1984).

The efferent supply consists of about 1,800 neurones and divides into two components; the lateral and the medial efferents (Warr, 1978, figure 1.16). About 1,000 of these are lateral efferent fibres that branch off to synapse with the IHC Type I afferent fibres. Thus, one efferent fibre supplies many afferents in any given region (Spoendlin, 1988). The efferent fibres also act to reduce the activity of the afferent fibres, and may consequently have a role in modulating against excessive metabolic activity in these fibres (Comis, 1970, Ruel *et al*, 2001). The remaining 800 or so medial efferents innervate the OHCs, and again significant branching occurs with one efferent supplying up to 25 OHCs. The physiological role of the efferents is to decrease sensitivity of the OHCs to presumably provide some minor degree of protection against noise damage (Wiederhold, 1970).

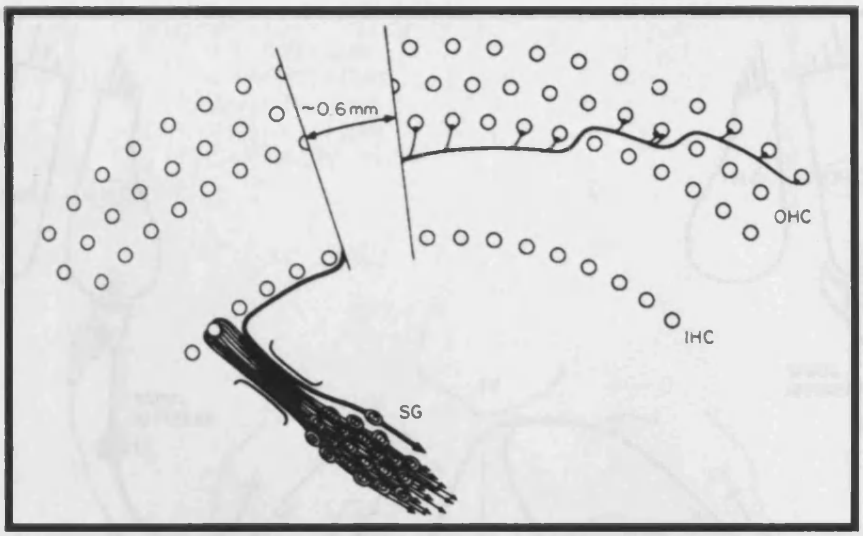


Figure 1.15. A schematic diagram of auditory nerve fibre connections. The greatest majority of type I fibres synapse with IHCs whereas a few fibres (Type II) synapse with OHCs after running basally for about 0.6mm. SG – Spiral ganglion. Taken from Spoendlin, 1978.

The electrophysiological response of CN fibres has been carried out by using very fine tipped (about 0.3μM) 2.7 M K^+Cl filled glass microelectrodes (Evans, 1972 and Kiang *et al*, 1965). These were used to record the temporal patterns of action potential discharge activity in each nerve fibre in response to precisely controlled discrete acoustic stimuli that were varied in frequency and intensity. The changes in discharge rate to stimuli were compared with reference to the spontaneous activity in each fibre to plot response curves. An example of one of these plots is shown in figure 1.10.

Individual CN fibres essentially follow the physiological response of the prior elements of the cochlea to acoustic stimuli. That is, they reflect the sensitivity and selectivity to stimuli of the tonotopic point of the cochlea which they innervate. This is turn reflects the *integrated* activity of the BM, OHCs and IHCs.

The fibres essentially act as passive conduction elements in coding for stimuli frequency. They do however introduce a further level of signal processing in coding for stimulus intensity. This is dependent on the position of the fibre relative to the IHC and the pharmacology of glutamate excitation.

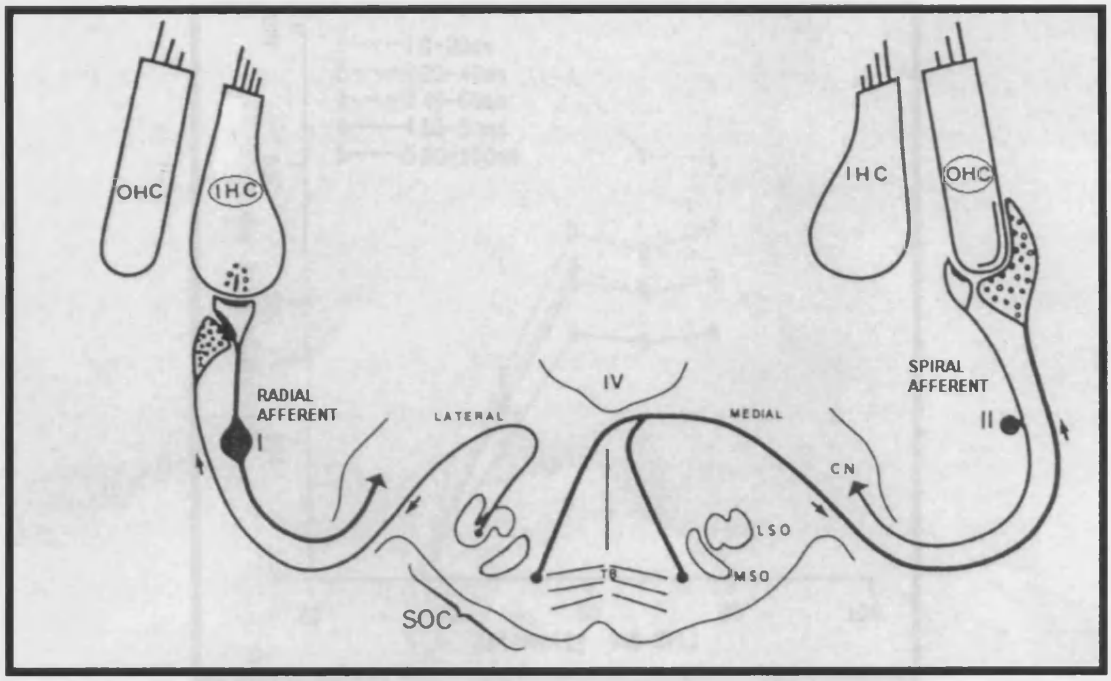


Figure 1.16. Schematic drawing of the neuronal connections of the IHC and OHCs. The left side shows the connections to the IHCs via the predominant type I radial afferent and the lateral efferent. The right side shows the OHCs with the greatly reduced type II spiral afferent connections and medial efferents (adapted from Puel, 1995).

1.4.1. Spontaneous CN activity

There is large variation of SR in guinea pig CN fibres ranging from about 0 to 130 spikes/sec (Cooper, 1989 and Mulheran, 1990) shown in figure 1.17. The frequency histogram for SR is bimodal in appearance with low SR discharging between 0 to 25 spikes/sec and high SR fibres discharging between 25 to 130 spikes/sec. The temporal discharge pattern is quasi-Poisson with a normal interspike mode of 3 to 7 ms (Mulheran, 1990). This reflects the Poisson probability of transmitter release by the IHC, which is modified by the relative refractory properties of the nerve. The absolute refractory period of guinea pig CN fibres is about 0.8 to 0.9 ms, and both the relative and absolute values reflect the kinetics of the refractory properties of the Na^+ channel.

Liberman (1982) found that the location of the CN fibre with respect to the IHC was related to SR. Those fibres with a high SR would synapse with the IHCs on the outer side, ie nearest the OHCs, whilst those that had a low SR would synapse on the inner side of the IHC, ie nearest the modiolus.

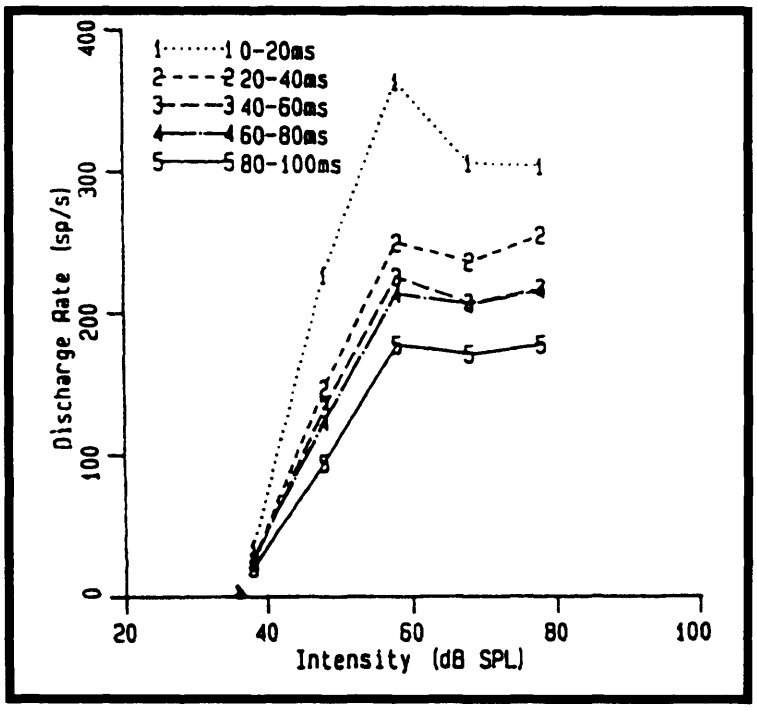


Figure 1.17. The mean activity (discharge rate) of a CN fibre measured at approximately 8 kHz. Data are based on approximately 4000 spikes recorded at each stimulus. Note the very high discharge rate within the first 20ms of stimulus (as indicated by number 1 on the plot) and then a stabilisation of activity over time. Adapted from Cooper (1989).

1.4.2. Driven CN activity - frequency and intensity coding

The afferent response of CN fibres to stimuli is always excitatory, never inhibitory (Evans, 1972). However, reduction in spontaneous and driven output can be mediated by the efferent system synapsing on both IHCs and CN fibres (Comis 1970, Ruel *et al* 2001).

The frequency tuning curve is often used to provide a direct measure of the selectivity characteristics of a fibre. This is done by recording an increase in discharge rate just above SR (Kiang *et al*, 1965 and Evans, 1972). This plot is very similar to the isovoltage tuning curves gained from OHCs and IHCs as shown in Figure 1.13 (Cody and Russell, 1978). This tuning curve plot effectively reflects the two components of the passive and active model discussed earlier. The lower frequency 'tail' reflects the OHC modulated passive resonance properties of the cochlear partition, whereas the sharply tuned tip reflects the frequency specific amplification of the active process described in section 1.3.1. This tip is particularly sensitive to trauma and reflects damage from each of the previous transduction steps (Liberman, 1984 and Liberman *et al*, 1984a-c). The tip of the tuning curve gives the threshold of the fibre and the frequency at this point is referred to as its characteristic frequency or CF (Kiang *et al*, 1965).

The cochlea is able to code for an intensity range from 0 to over 120 dB SPL (Evans and Palmer, 1980). This reflects the range of displacement of the cochlear partition, which in turn drives the OHCs and IHCs. The input/output function of CN fibres in turn defines the neural coding of intensity. This again has been shown to correlate with high SR fibres saturating at about 30-40 dB above threshold (Evans and Palmer, 1980 and Winter, 1990). Those fibres with SRs between 1-20spikes/sec showed sloping saturation with a range of about 40 to 50 dB. Very low SR fibres, however, did not appear to saturate. The thresholds of these fibres also correlate with SR, with low threshold fibres being the most sensitive and low SR fibres being the least sensitive. The summated activity of all fibres firing in response to coherent discrete stimuli is measured in the compound action potential (CAP) measured using gross recording electrodes (section 1.5).

1.5. Gross cochlear potentials

Making microelectrode recordings from the CN, the IHCs and OHCs *in vivo* presents considerable technical difficulties, and relatively few laboratories worldwide perform these measurements. Fortunately, a great deal of useful information can be gained from the cochlea and the CN, by making electrophysiological measurements using gross recording electrodes (Pickles, 1988). The recording principles and techniques are essentially the same as those applied elsewhere in the periphery and the CNS. The tip of the gross recording electrode is approximately 100 to 200 μM and is able to integrate electrical signals generated over a few mm at the point of contact. Attenuation of signal strength is generally exponentially proportional to the distance from the electrode (Beagley, 1979).

Gross measurements from the cochlea have revealed a number of distinct components to the potential and these were later identified as being generated by the different neurosensory components in the cochlea (Pestazolli and Davis, 1956, Goldstein and Kiang, 1958, Pickles. 1988).

The principle components to the compound action potential (CAP) arising from the cochlea and CN are shown in figure 1.18. Normally, discrete tone 'pips' of about 5 ms in duration are used to generate a coherent, synchronised response from a specific frequency region along the cochlear partition. This synchronous response is then differentially amplified x 10,000 and averaged between x 10 to 100 to reduce background noise.

Depending on stimulus intensity, the resultant CAP trace is usually between 1 and 500μV in amplitude.

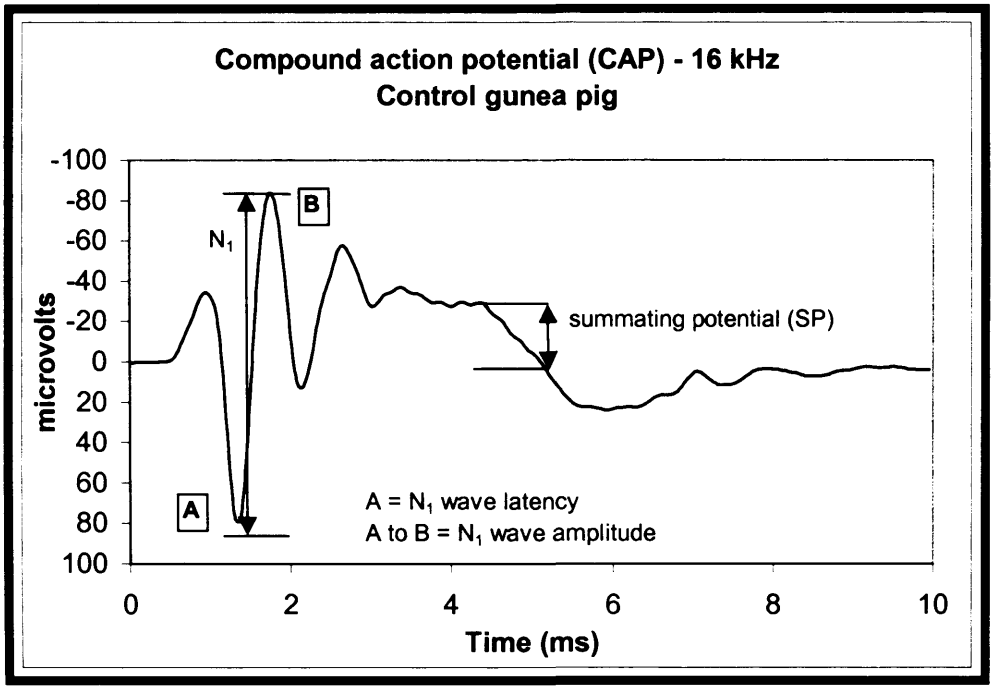


Figure 1.18. The compound action potential taken from a control guinea pig following a 16 kHz tone pip stimulus (80dB). The measurement criteria for the N_1 wave latency and amplitude are shown. The SP is also shown for interest.

The sinusoidal component of the CAP in response to simple tonal stimulus (not usually seen on an averaged trace) is the cochlear microphonic or CM. This is generated by the OHCs to stimulus, and reflects K^+ currents as they move from the scala media though the sound modulated variable resistance provided by the OHCs and through into the scala vestibuli (see section 1.3.2). The response is not a truly tonotopic representation of OHC activity and is subject to complex spatial, and phase generator relationships of OHCs in the cochlea. The CM signal tends to be dominated by contributions from OHCs in the basal part of the cochlea. This makes it a less specific measure of any traumatic effects that may occur in OHCs alone (Patuzzi *et al*, 1989).

The dc shift in the CAP is the summating potential (SP) which reflects evoked steady state K^+ currents generated by IHC and OHCs as described in section 1.3 and shown in figure 1.18. The generation of this potential is more frequency specific, at least for the IHCs, and provides a better representation of the effects of cochlear trauma on IHC/OHC function. The experimental utility of the SP is limited however by this joint generation by IHC/OHC *dc* currents (Dallos *et al*, 1972, 1985). It is further limited by its overall polarity being both intensity dependent and on the relative contributions made by IHCs

and OHCs at a given tonotopic point. This potential for bidirectionality of response can sometimes make the SP difficult to interpret so that sometimes the SP will be negative (Puel *et al* 1988, 1994).

The clear negative going potential in the CAP in figure 1.18 is referred to as the N_1, so called as it represents the first negative peak of the cochlear CAP. It consists of the synchronised summated activity of the hundreds of afferent nerve fibres synapsing with the IHCs at the tonotopic location activated by the tone 'pip' stimulus (Pestazolli and Davis, 1956, Goldstein and Kiang, 1958, Pickles, 1988). This relative negativity is due to the mass movement of Na^+ ions passing through the voltage dependent Na^+ channels of the afferent fibres.

The amplitude and latency characteristics of the N_1 signal represents a direct measure of activity of the stimulus response of the OHC - IHC – afferent synapse – afferent nerve signal path. Whilst the generation of the N_1 does not provide sufficient resolution of the potential site(s) of action of cochlear trauma, its monopolarity and intensity dependence as the last link in the transduction chain, leads to its universal use in the literature as a marker of cochlear function in pharmacological studies (Puel *et al*, 1988).

1.6. The measurement of spontaneous CN activity

The CM, SP and N_1 of the CAP represent compound evoked potentials generated by the separate functional components of the cochlea. These can all be measured at the cellular level using microelectrodes. Spontaneous activity of the CN is an important feature of neural activity that until recently did not have a gross recording equivalent of the above evoked signals. By definition, spontaneous activity is not evoked but is still a key parameter of both normal and experimental pharmacology in the cochlea.

A number of workers have however, employed the commonly used engineering technique of the Fast Fourier Transform (FFT) to provide a measure of compound spontaneous activity using gross electrode signal (Dolan *et al* 1989, 1990, Cazals and Huang 1996, Lima da Costa *et al.* 1997). This technique takes the varying voltage/time signal and breaks it down into its component amplitude/frequency. The resulting signal spectrogram is usually expressed in terms of Volts power vs Hz as shown in Figure 1.19 and is referred to in this thesis as ensemble spontaneous CN activity or ESAC. This technique was developed by the French mathematician, Joseph Fourier (1768-1830).

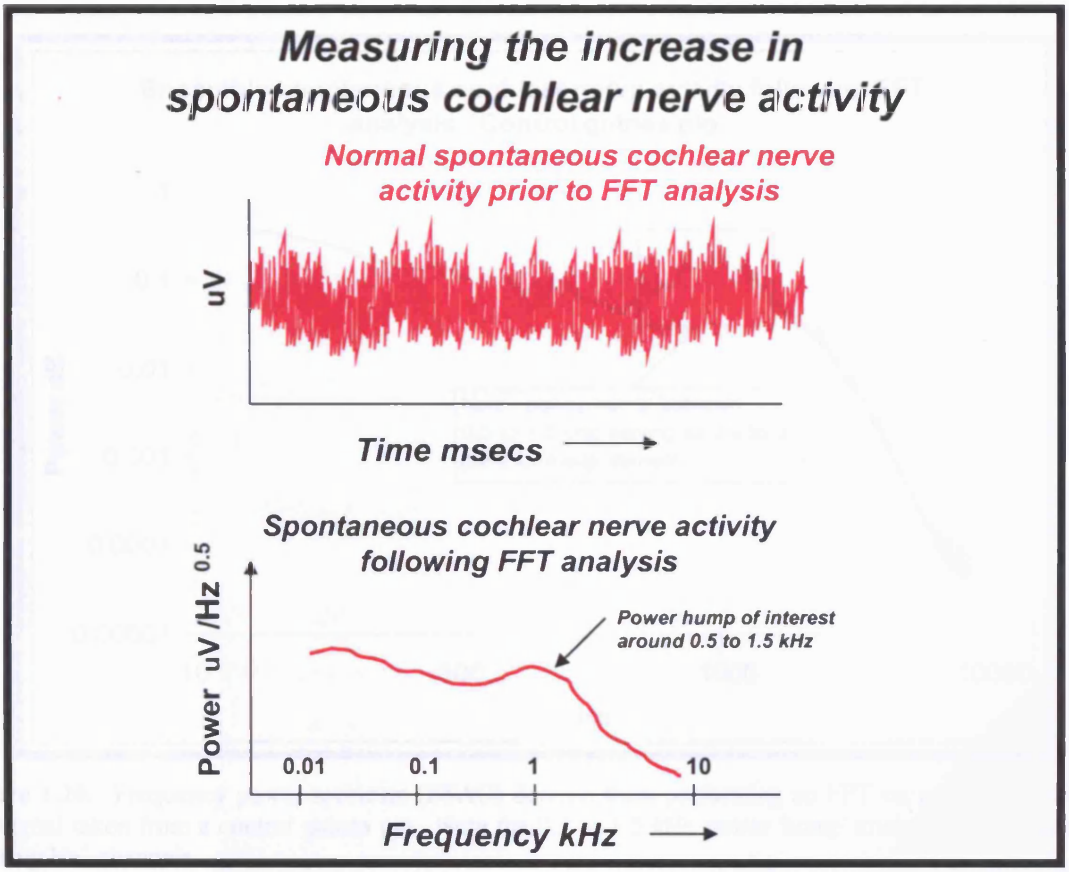


Figure 1.19. An illustration showing the measurement of normal spontaneous CN activity and its subsequent conversion (FFT) enabling power quantification.

A brief description of the process is given below, but the mathematical theory behind the FFT transform is covered in detail elsewhere (Meade and Dillon, 1991). The varying time signal is first taken and digitally sampled at a specific time interval that allows adequate detail and resolution of the signal to be seen in its digital representation. The digital sampling frequency that determines resolution is set by the Nyquist criteria (Meade and Dillon, 1991). This states that for a given upper *signal* frequency, the sampling frequency must be a least twice the signal frequency of interest. In this case, the sampling frequency was 16 kHz, which would allow a resolution up to 8 kHz. The next stage of the transform compares the relative amplitudes of each sample and how frequently this amplitude occurs.

Normally, when amplified and displayed, the constant spontaneous activity of all CN fibres is reflected as a noise signal on screen as shown in figure 1.19. When unfiltered, this signal is typically about 10 μV p-p. If this signal is subject to an FFT, it transforms the results into a frequency/power spectrum plot as illustrated in figures 1.19 and 1.20. In this thesis, ensemble spontaneous CN activity is termed ESAC, or EDAC in the driven state (ie during noise insult).

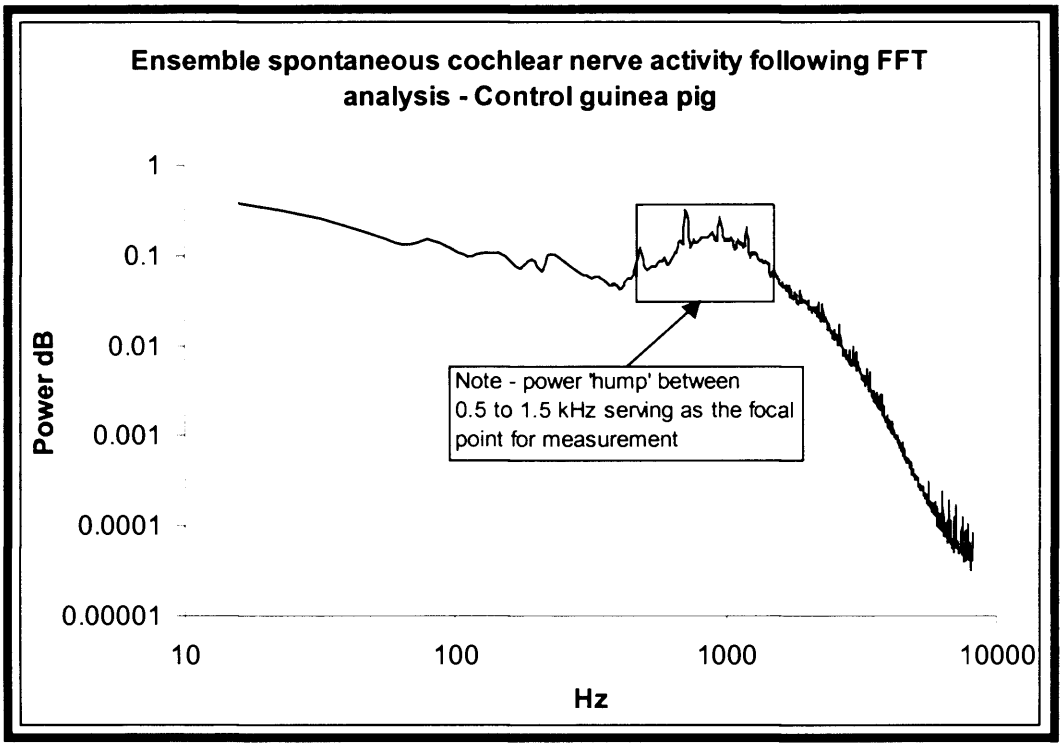


Figure 1.20. Frequency power spectrum (ESAC) derived from performing an FFT on gross spontaneous CN signal taken from a control guinea pig. Note the 0.5 to 1.5 kHz power 'hump' arising from the activity of many Na^+ channels.

Given the high SR of the afferent fibres (Mulheran, 1990) the greatest contributor to the biological electrical noise signal would be expected to be the CN. This has been shown to be the case by the use of intracochlear kainate perfusion, an excitotoxic glutamate agonist, and the Na^+ channel antagonist tetrodotoxin. Both agents resulted in the signal declining rapidly and exponentially (Dolan *et al*, 1990, Fergie, 2002 unpublished data). This signal has been shown to be physiologically vulnerable and modulated by anaesthetic depth, efferent activity and anoxia. Moreover, the power also increases in a parallel manner to the growth of the gross CAP N_1 (Dolan *et al* 1990, Cazals and Huang 1996, Lima da Costa *et al* 1997). In its overall appearance, the spectrum above about 500 Hz is similar to that obtained for an individual action potential spike. In particular, the 1 kHz hump reflects the opening and closing kinetics of the Na^+ channel. The higher frequency components (up to 10 kHz) reflect the rapid opening phase of the channel (Dolan *et al* 1990, Lima da Costa *et al* 1997).

The application of this technique provides a novel way of investigating both the gross spontaneous activity of the CN, and the physiology and pharmacology of Na^+ channel behaviour. Importantly, these findings are not just strictly limited to the CN and can be extrapolated to any part of the CNS. In this study, this technique was used as a way of

establishing the pathophysiological effects of noise and the action of the voltage dependent Na^+ channel blocker lamotrigine as shown in chapter 5.

1.7. Cochlear pharmacology

Much of the work in understanding the pharmacology of the afferent and efferent system in the cochlea has been carried out by the Montpellier-based group, lead by Pujol and Puel, and by Gil Loyzaga in Madrid. Throughout the last two decades comprehensive characterisation of glutamate as the afferent transmitter in the cochlea has been achieved by Puel *et al* (1991, 1994, 1995, 1998, 1999). This work involved immunohistochemical, ultrastructural, pharmacological and electrophysiological techniques.

The work established that the afferent IHC synapse shares many features of glutamate pharmacology as found in the rest of the nervous system. This was principally determined by use of glutamate receptor agonists and antagonists in experiments investigating excitotoxicity in the guinea pig cochlea. The general features underlying glutamate pharmacology and excitotoxicity are outlined below.

1.7.1. Glutamate receptors in the cochlea

Cochlear glutamate receptors (GluRs) include the two major groups; ionotropic receptors which are ligand gated cation channels, and metabotropic receptors which are G-protein coupled receptors (Jorgensen *et al*, 1995). This is shown in figure 1.21. The receptors of particular interest in understanding acute damage following noise exposure are the ionotropic receptors, which carry transducer currents that lead to generation of neural action potentials.

The glutamate receptors in the cochlea are named after popular agonists (Jorgensen *et al*, 1995, Parks, 2000):

- NMDA (N-Methyl-D-aspartate)
- AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)
- KA (kainic acid or kainate).

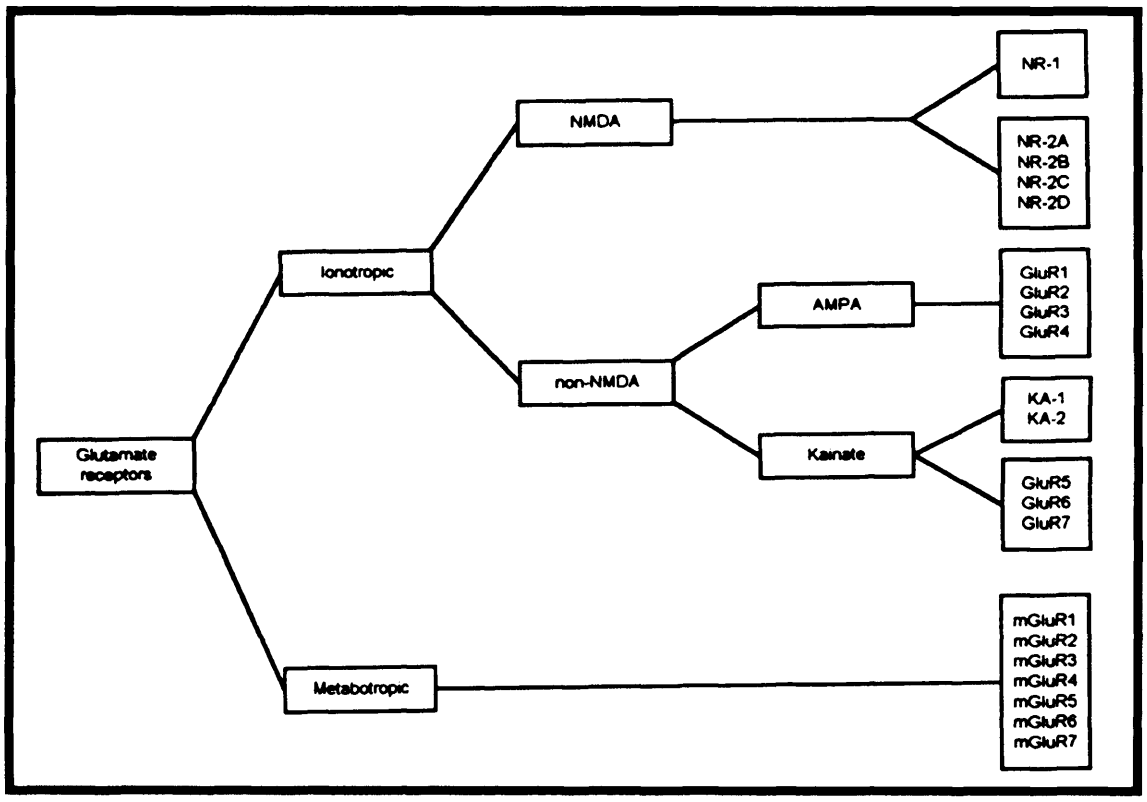


Figure 1.21. Cloned glutamate receptor subtypes (taken from Jorgensen *et al*, 1995)

1.7.1.1. Non-NMDA ionotropic receptors – AMPA and KA

When glutamate forms a ligand with the AMPA/KA receptors, Na^+ current is carried into the postsynaptic terminal to depolarise the axon. Within the inner ear, these receptors are activated *before* NMDA receptors by low and moderate sound intensities. This parallels their activation and 'fast' current carrying characteristics elsewhere in the CNS (Jorgensen *et al*, 1995). The most commonly used antagonists in functional studies of non-NMDA receptors in the auditory system are the quinoxalinediones DNQX and CNQX (Puel *et al*, 1991, 1994).

1.7.1.2. NMDA ionotropic receptors

The NMDA receptor is directly coupled to an ion channel. When the glutamate ligand forms, depolarising cation current is carried by Na^+ and Ca^{2+}. The NMDA receptor is distinct from the AMPA/KA receptors in that a number of additional criteria need to be satisfied prior to its activation, apart from the presence of glutamate. The neurone needs to be sufficiently depolarised in order to relieve a voltage dependent magnesium block of the channel. Glycine also needs to be present to activate the NMDA channel.

Functionally, the NMDA channel is considered to carry current resulting from more intense stimuli. Puel (1995) quotes a figure of 80 dB SPL being required to activate these

receptors. The current kinetics are also 'slower' contributing to later excitatory post synaptic potential (EPSP) current. NMDA currents are also considered to be primarily responsible for causing excitotoxic damage, primarily due to the influx of Ca^{2+}.

1.7.2. Glutamate release, re-uptake and re-cycling in the cochlea

Glutamate is synthesised from glutamine by the mitochondrial enzyme phosphate activated glutaminase (PAG). It is stored in synaptic vesicles in the IHCs, and is also present in high concentrations in the cytoplasm. The rate of release of the synaptic vesicles is dependent on the IHC intracellular Ca^{2+} concentration as described above in 1.3.2. As there is usually a substantial standing Ca^{2+} current into the IHCs, there is a large release of glutamate in the absence of stimuli which drives the spontaneous activity of CN fibres (Kros and Crawford, 1988).

Unlike acetylcholine (ACh), glutamate is not enzymatically de-activated and has to be rapidly removed from the synaptic cleft. If this removal is compromised this may lead to excitotoxicity (Bruno *et al*, 1993). Release and uptake via glia cells and neurones is via a high affinity Na^+-dependent transport system. This Na^+ dependent glutamate transporter (GLAST) in the cochlea has been shown by immunolabelling to be present in high concentration in the supporting cells around the IHCs (Furness and Lehre, 1997). The normal functioning of this carrier is important in avoiding excitotoxic damage. Under conditions of metabolic stress, the GLAST transporter actually reverses its direction of transport and carries both Na^+ and glutamate back out into the synaptic cleft (Kiedrowski *et al*, 1994a,b). This property was considered to be important in the development of the noise/hypoxia (NH) model utilised in this thesis.

Once taken up by the supporting cells, glutamate is converted into glutamine via the enzyme glutamine synthetase, and the glutamine is then taken back by the glutamatergic neurons (figures 1.22 and 1.23). Radioautographic studies have indicated that a similar cycle for glutamate metabolism is also apparent in IHCs (Eybalin, 1983).

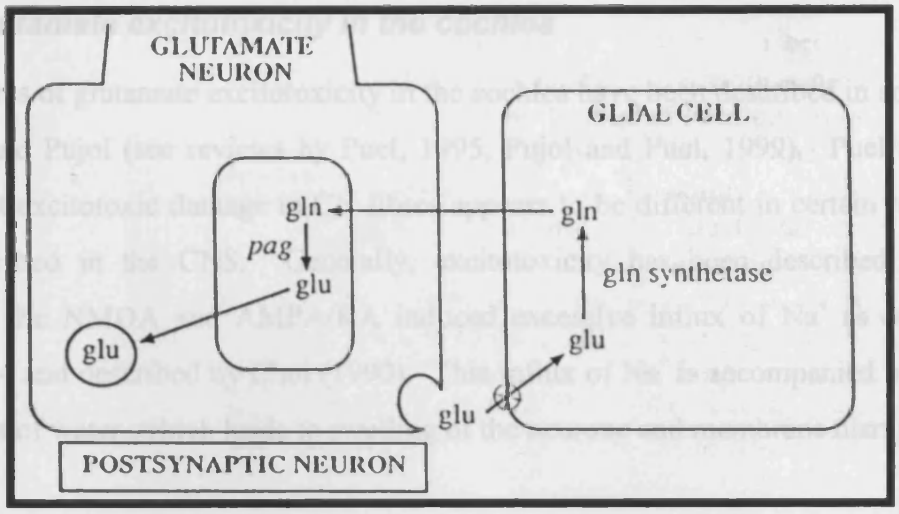


Figure 1.22. An overview of the neuronal-glial glutamate cycle. Glutamate (glu) is synthesised from glutamine (gln) by the mitochondrial enzyme phosphate-activated glutaminase (pag) and is then stored in vesicles prior to release. On release, reuptake is via glial cells and neurons by a high affinity Na^+ dependent transport system where glutamine synthetase converts glu back to gln where it is taken up by the neurons. Taken from Dawson *et al*, 1995.

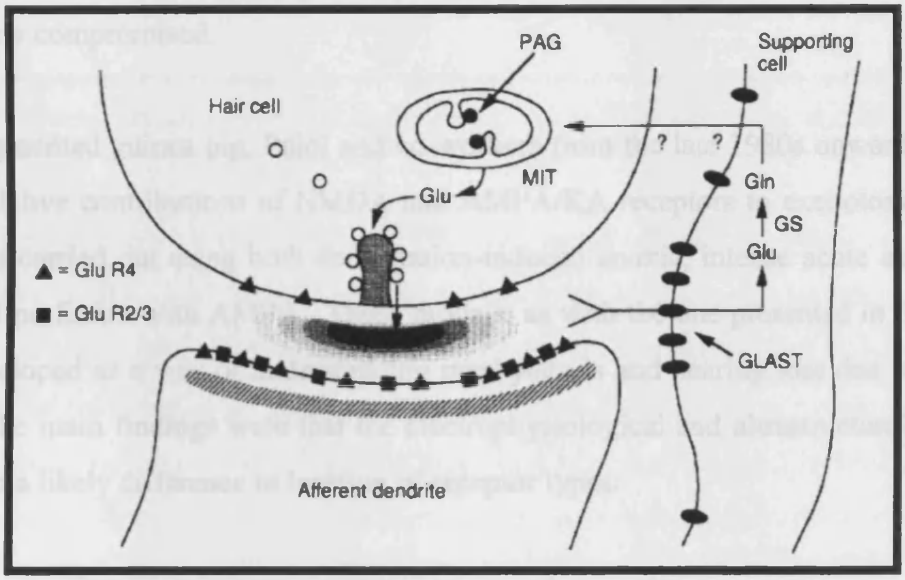


Figure 1.23. A simplified diagram illustrating the potential mechanisms for glutamate clearance and metabolism at afferent synapses within the inner ear. Glutamate is released by excocytosis from the site of the presynaptic body (localised on the hair cell) onto a post synaptic receptor matrix containing AMPA receptors (the afferent dendrite). This is density dependent as represented by the hatched area. The supporting cell shown on the right provides a reuptake mechanism for glutamate (denoted by ovals) where it is converted to glutamine (Gln) by glutamine synthetase (GS) and then transferred back into the hair cell by an unknown mechanism. Glutamine is then converted back into glutamate by the enzyme phosphate-activated glutaminase (PAG) which is found in the mitochondria. Vesicular accumulation of glutamate then occurs ready for the next exocytotic event. Taken from Dawson *et al*, 1995.

Therefore the concentration of glutamate in the synapse is a balance between the rate of release from the presynaptic terminal and the rate of re-uptake. The affinity of the receptors for glutamate and their deactivation and desensitisation kinetics control the time course of synaptic currents produced by the transmitter available in the synaptic cleft.

1.7.3. Glutamate excitotoxicity in the cochlea

The features of glutamate excitotoxicity in the cochlea have been described in some detail by Puel and Pujol (see reviews by Puel, 1995, Pujol and Puel, 1999). Puel and Pujol report that excitotoxic damage in CN fibres appears to be different in certain respects to that described in the CNS. Generally, excitotoxicity has been described as firstly involving the NMDA and AMPA/KA induced excessive influx of Na^+ as outlined in figure 1.24 and described by Choi (1990). This influx of Na^+ is accompanied by osmotic movement of water, which leads to swelling of the neurone and membrane disruption.

If excitotoxicity is severe, then Ca^{2+} entry principally via the NMDA gated channel may leads to the setting up of a cascade of intracellular signals that lead to apoptosis and necrosis (Choi 1990, Bruno *et al* 1993) shown diagrammatically in figure 1.24. Cell death is not inevitable if the mechanisms dealing with neuronal Ca^{2+} homeostasis are not excessively compromised.

In the pigmented guinea pig, Pujol and co-workers from the late 1980s onwards focused on the relative contributions of NMDA and AMPA/KA receptors to excitotoxicity. This work was carried out using both decapitation-induced anoxia, intense acute exposure to noise and perfusion with AMPA. These models, as with the one presented in this thesis, were developed as a way of understanding presbyacusis and hearing loss due to auditory insult. The main findings were that the electrophysiological and ultrastructural evidence pointed to a likely difference in location of receptor types.

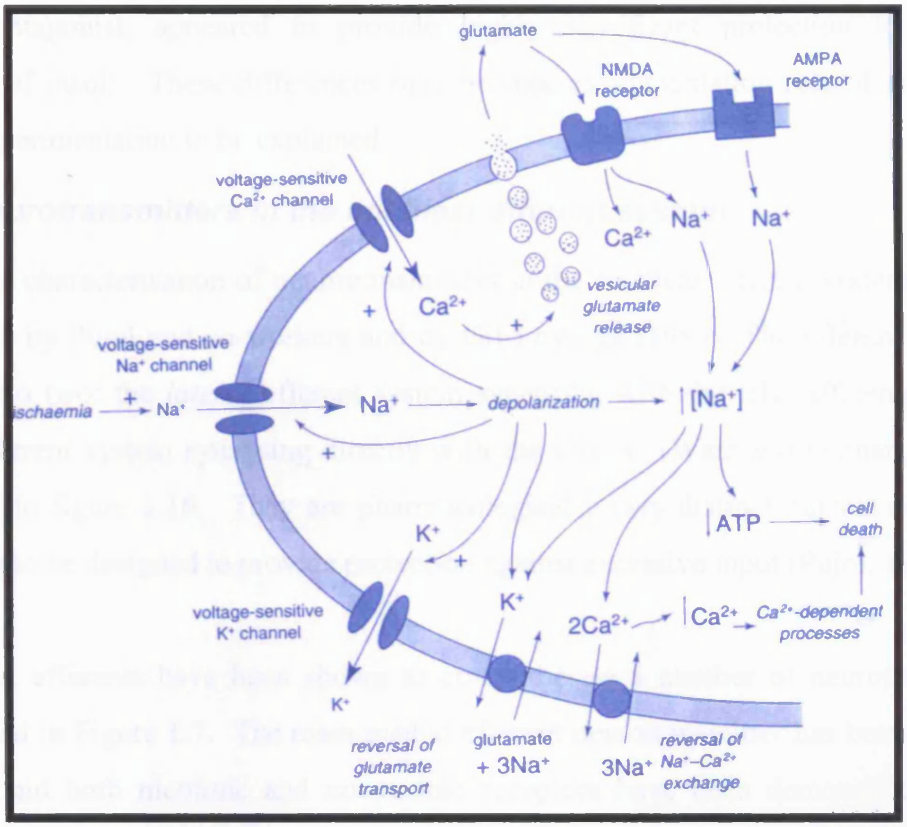


Figure 1.24. A schematic representation of the role of Na^+ and Ca^{2+} channels during an ischaemic episode. Taken from Taylor and Meldrum, 1995.

This evidence fitted with the location of the most sensitive neurones being on the pillar, or OHC side of IHC, and the less sensitive ones being on the modiolar side of the IHC Liberman (1982). The most sensitive fibres synapses are considered only to express AMPA receptors (Puel *et al,* 2002). Moreover, these channels do not carry appreciable Ca^{2+} currents (Ruel *et al,* 2000). These fibres were also not protected by blocking of the NMDA receptor with D-AP5 prior to anoxia, but were fully protected by pre-treatment with DNQX, an AMPA antagonist (Puel *et al*, 1991).

NMDA receptors do appear to be expressed in the less sensitive CN fibres on the pillar side and additional protection to that by DNQX was afforded in the anoxia model by pretreatment with D-AP5 (Pujol *et al*, 1992, 1993). The authors also demonstrated that after 10 minutes cochlear perfusion with 200μM AMPA, the severely damaged CN fibres showed significant signs of regeneration after 5 days (Puel *et al*, 1995, 1999). This shows that the apoptotic pathway had not been followed in this model.

The work appears to be in some contrast at least with the NH models in the rat and guinea pig (Chen *et al*, 2001, Selvadurai *et al*, 2000). In these models, the use of MK-801, an

NMDA antagonist, appeared to provide highly significant protection to differing severities of insult. These differences may be species/pigmentation related and require further experimentation to be explained.

1.7.4. Neurotransmitters in the cochlear efferent system

Systematic characterisation of neurotransmitters in the cochlear efferent system has been carried out by Pujol and co-workers and by Gil Loyzaga (1989). The efferent system is divided into two: the *lateral* efferent system synapsing with the IHC afferents, and the *medial* efferent system synapsing directly with the OHCs (Warr and Guinan, 1979) as illustrated in figure 1.16. They are pharmacologically very distinct but physiologically both seem to be designed to provide protection against excessive input (Pujol, 1994).

The lateral efferents have been shown to co-synthesise a number of neurotransmitters summarised in Figure 1.7. The main medial efferent neurotransmitter has been shown to be ACh, and both nicotinic and muscarinic receptors have been demonstrated in the OHCs (Pujol, 1993). Gamma aminobutyric acid (GABA) and calcitonin gene related peptide (CGRP) have also been identified, though their roles as possible neuromodulators is less well defined. In contrast, ACh has been demonstrated to have a protective role by attenuating OHC electromotility and thus affecting cochlear micromechanics (Wiederhold, 1986). Of these neurotransmitters, most prominence and recent research interest has involved GABA and dopamine. Recently, dopamine has been shown to decrease both SR and driven activity in single CN fibres (Ruel *et al*, 2001). Moreover, a number of dopamine (D_2) receptor agonists have been shown to protect against excitotoxicity, presumably by their ability to also reduce driven activity (Puel *et al* 1993, Ruel *et al* 2001). It has also been proposed that dopamine signalling may be involved in the regeneration of damaged synapses (Pujol, 1994).

Whilst the efferent system plays an important role in modulating cochlear function it is clear that its protective is capacity is overcome in animal models of noise trauma as reviewed in detail in Chapter 4. In humans, the protective effect is likely to contribute to the ability of the cochlea to withstand insult over the years. Unfortunately, this too is eventually overcome as confirmed by the number of people presenting with varying degrees of noise induced auditory pathology (Davis, 1983).

Chapter 2. Experimental Methods

2.1. Principle experimental groups

Three main groups of experiments were carried out within this thesis using the albino guinea pig as the test species. Each set of experiments had specific technical variations designed to investigate the following:

1. The effects of reduced oxygen (hypoxia) over 18 to 12% atmospheric oxygen, noise and a combination of the two on the brainstem auditory evoked response (BAER) using different sedation/anaesthesia regimens.

2. The effects of i) sedation/anaesthesia and ii) round window perfusion of artificial perilymph on compound action potentials (CAP) and ESAC/EDAC.

3. The effects of hypoxia, noise and a combination of the two on CAP and ESAC/EDAC – defining an acute model of hearing loss.

4. The ability of the antiepileptic lamotrigine (LTG) to act as a cochleoprotectant following acute noise exposure as assessed by CAP (threshold and input output function) ESAC and EDAC.

Specific details of experimental paradigms used above are covered in the relevant chapters, but the general experimental methods are given below.

2.2. Animal Husbandry

A total of 124 healthy adult male albino guinea pigs (Harlan UK, Limited) weighing between 200 and 620g were used. All experiments were carried out in accordance with Home Office Regulations as described in The Animals Act (Scientific Procedures) 1986 and covered by the appropriate Personal, Project and GSK Site Home Office Licenses.

Animal husbandry was standard as detailed in relevant GSK SOPs (in particular SOP/WPT/4, 31, 34, 38, 79 and 97). Animals were randomly allocated to cage and group by hand, and housed in groups of up to six in solid bottomed cages. Fresh sawdust, hay, fruit and vegetables were supplied at least every 3 days.

Water from the domestic supply was changed daily and guinea pig pellet diet, FD1 SQC supplied by Special Diet Services (SDS), Ltd., UK was given *ad libitum.* Temperature was maintained between 16-20°C and humidity between 45-70%. Lighting was on a 12 hour dark/12 hour light cycle (light from 7am to 19.00 hours, then dark until 7am).

It was considered improbable that any substance likely to be absorbed from the diet and drinking water provided for the animals would prejudice a meaningful evaluation of the results.

2.3. Surgical procedure

Prior to sedation or surgery, animals were weighed to calculate the correct dose of sedative or subsequent drug treatment. The area around the left pinna was shaved and an otoscopic examination of the ear canal was performed to confirm the absence of infection or cerumen.

2.4. The use of sedation/anaesthesia in the experimental design

It is important to emphasise that any form of surgical anaesthesia should satisfy the criteria for good analgesia, muscle relaxation and loss of reflex activity. In part, these criteria should be fulfilled by a wide safety margin and long duration of action. Animals should be subjected to minimal metabolic or physiological disturbance if successful experimental techniques are a prerequisite. Consideration should also be given to ease of administration, with stress and discomfort kept to a minimum. Guinea pigs are historically one of the most difficult species to anaesthetise both with injectable and inhalation agents (Cannell, 1972). One of the main problems is achieving the required level of sedation without compromising respiratory or cardiac function (bradycardia).

Three anaesthesia/sedation regimens were assessed in this thesis. The overall aim was to determine a suitable regimen that would allow surgical intervention and repeat-measure auditory recordings, without compromising animal physiology. In all regimens, loss of the pedal reflex was used to determine sufficient sedation. The parenteral agents described below were used as comparators to the inhaled gaseous agent Isoflurane in order to gain an insight into their actions on cochlear responses. An additional benefit from the parenteral agents was that neither agent required the placement of a facemask nor the use of obtrusive scavenging apparatus (used for gaseous anaesthetics) which could

hinder the experimental set up. A brief introduction to the agents used in the three regimens is given below.

- **Regimen 1 - BAER recordings.** An intramuscular injection of 0.6mL/kg fentanyl/fluanisone (Hypnorm®) administered into the upper hind limb muscles. This light sedative regimen was sufficient for the superficial placement of subdermal recording electrodes but not surgical procedures. Supplementary doses were administered if the animal showed minimal signs of arousal during the experiment (ie limb twitch or chewing).

- **Regimen 2 - BAER recordings.** An intramuscular injection of 0.6mL/kg fentanyl/fluanisone (Hypnorm®) administered into the upper hind limb muscles followed by a 2mL/kg intraperitoneal injection of midazolam (Hypnovel®). This light sedative regimen was also sufficient for the superficial placement of subdermal recording electrodes but not surgical procedures. Supplementary doses were administered as in Regimen 1.

- **Regimen 3 - BAER, CAP and ESAC/EDAC recordings.** Injections of 0.5mL/kg medetomidine (Domitor® - intramuscular) and 6mL/kg fentanyl (Sublimaze® - intraperitoneal) approximately 10 minutes later. Supplementary exposure to 5% Isoflurane gas mixed with oxygen at a rate of 1.5L/minute was necessary for gaining access to the cochlea and during the period of surgery only. Supplementary parenteral doses were administered following a strict time protocol of every 15 minutes. This third regimen was used exclusively throughout chapters 4 and 5.

2.4.1. Isoflurane

Isoflurane, a halogenated methyl-ethyl-ether and an isomer of enflurane (Larsen and Langmoen, 1998) was primarily chosen because it is an established and safe method of anaesthesia for small animal surgery within GSK. It also offers rapid onset of duration and action on the central nervous system, with good post-surgical recovery. However, its use was limited to induction for acute ear surgery only thereby reducing the potential for any problems of excessive CNS depression (Larsen *et al,* 1994).

2.4.2. Fentanyl/fluanisone (Hypnorm®)

Neuroleptanalgesia is a term used to describe the combination of a potent sedative analgesic agent with a major tranquiliser to induce a state of central nervous system depression bordering on general anaesthesia (Green, 1975). A combination of fentanyl/fluanisone ((Hypnorm®) is widely used for the induction of neuroleptanalgesia in small laboratory animals.

Fentanyl (Sublimaze®) is a highly potent and efficacious μ opioid agonist similar in action to morphine but 20 to 100 times more active in terms of analgesia (Green, 1975). It can cause profound central nervous system and respiratory depression as well as bradycardia. Fentanyl takes effect very rapidly buts its duration of action is relatively short

Fluanisone is a major tranquiliser belonging to the butyrophenone group, with putative antipsychotic activity and dopamine antagonist properties. It potentiates the analgesia provided by fentanyl and also antagonises any respiratory depression (Inoue *et al*, 1994). The combined actions of fentanyl and fluanisone are therefore longer in duration due to this potentiation.

2.4.3. Midazolam (Hypnovel®)

Midazolam is a water-soluble benzodiazepine and has a similar profile of activity to Diazepam except it is more rapid in onset and has a shorter duration of action due to its rapid metabolism (Flecknell and Mitchell, 1984). It has been shown that midazolam on its own does not induce anaesthesia in rodents even at doses as high as 30mg/kg but it has been shown to potentiate the effects of fentanyl and droperidol (Pieri *et al*, 1981).

Results obtained by Flecknell and Mitchell (1984) showed that fentanyl/fluanisone and midazolam combined produced neuroleptanalgesia with good skeletal muscle relaxation in mice, rats, gerbils, hamster, guinea pigs and rabbits. Previous work by Mertens and Muller-Deile (1991) has also shown that fentanyl combined with the benzodiazepine diazepam is a safer anaesthetic protocol in the guinea pig as in ensures definitive anaesthesia without respiratory depression and, unlike pentobarbitone, it does not suppress the BAER or CAP thresholds.

2.4.4. Medetomidine (Domitor®)

Domitor (medetomidine hydrochloride) is a synthetic α_2-adrenoreceptor agonist with sedative and analgesic properties (Mizobe and Maze, 1995). It is an ideal premedicant prior to surgical intervention due to its anxiolytic properties, which makes it an especially suitable agent in the guinea pig. The pharmacological restraint and pain relief provided by medetomidine facilitates animal handling, and aids in the conduct of diagnostic or therapeutic procedures. It also facilitates minor surgical procedures (with or without local anesthesia).

With medetomidine administration, blood pressure can be initially increased due to peripheral vasoconstriction and thereafter drops to normal or slightly below-normal levels. The initial vasopressor response is accompanied by a compensatory marked decrease in heart rate mediated by a vagal baroreceptor mechanism. The bradycardia may be partially prevented by prior (at least 5 minutes before) intravenous administration of an anticholinergic agent such as atropine. The administration of atropine was not required in this thesis as no evidence of an effect on heart rate was observed before during or after the experiments (as determined by pulse oximetry). Similarly, the reported effects on respiratory responses including an initial slowing of respiration and decreased tidal volume were not observed.

2.5. Monitoring and maintenance of general physiological function during experiments

After the induction of anaesthesia, the guinea pig was placed in a prone position (left ear upwards) in a purpose-built perspex chamber for the duration of all experiments. The chamber had a total volume of approximately 20 litres with a hinged lid and side door. The underside of the lid contained the drivers for noise generation and the side door allowed access for both surgery and anaesthetic apparatus. Various small side holes were available for electrode connections. There was one gas inlet near the bottom of the chamber which provided either stable or variable oxygenation (depending on the experimental paradigm) and one exhaust outlet valve near the top of the chamber (vented to the room ventilation system). After surgery and electrode implantation, the lid and side door were closed creating a low-noise recording environment. The average noise level within the chamber was typically less than 45dB SPL with the majority of frequencies below 1-2 kHz. This meant that this noise had little or no effect on threshold estimation of the higher frequencies (8-30 kHz) measured in this study.

Core body temperature was maintained at 37±1°C using a Harvard homeothermic control unit and blanket placed under the guinea pig with a silicone sealed rectal temperature probe (calibrated using a water bath and thermometer). A non-invasive pulse oximeter (Nonin 8500AV, Nonin Medical inc., Plymouth, USA) was placed on the forepaw to continuously monitor arterial blood oxygenation, which was maintained above 97% (unless hypoxia intervention was employed). Ventilation was maintained spontaneously throughout all experiments and the rate of breathing was visually monitored. Any changes in heart rate and breathing normally resulted in minimal blood/oxygen excursions below 97%. Supplementary oxygen was given until a stable reading was obtained. This procedure did not result in the rejection of any animal data. The pulse oximeter also gave a read out for heart rate which was kept within the normal range for the guinea pig in all experiments (180 to 350 beats per minute; Poole, 1999).

During BAER recordings only, electrocardiography (ECG) was performed using the Cardiovit A6 recorder. Prior to recordings, the guinea pigs flanks were shaved and highly conductive ECG electrolyte cream (Signa Creme) was used to ensure good conductance between the skin and the electrodes. Four subdermal needle electrodes were then attached to each flank. Recordings were produced at two paper speeds; 25 and 50mm/sec with heart rate (beats per minute) calculated automatically. All lead II ECG recordings were subsequently inspected manually for any abnormalities. The ECG was assessed at 15 minute intervals although noise stimulus was switched off during recording to avoid the potential for electrical interference. All measurements were fully documented throughout so that any unexpected excursions in cochlear physiology could be attributed to acute deficits in general physiological condition.

2.6. Surgical procedure

Following successful sedation as described previously in Regimen 3, a 3cm postauricular incision was made. The underlying muscles were reflected using sharp and blunt dissection to reveal the lateral surface of the bulla, with care taken not to lift the posterior meatal skin from the canal wall. The exposed bulla was then stripped of periosteum and the small amount of blood loss normally encountered was easily controlled with cotton wicks. A binocular operating microscope was then positioned over the guinea pig and, under low magnification, a hole was carefully made in the lower half of the bulla using a high speed mastoid drill (Citenco) and a 2mm cutting burr. This hole was enlarged to approximately 5mm in diameter using a small currette. The inner lining of the bulla was

then examined for any evidence of middle ear pathology, ie. where fluid was present or the mucosa was grossly inflamed. If this was the case, the experiment was terminated.

Careful manipulation of the animal allowed clear visualisation of the round window niche. Occasionally, the bony shelf-like projection from the lateral wall of the bulla over the round window was gently removed under high power magnification to allow better placement of the recording catheter (see section 2.7 and figure 2.1). Care was also taken not to disturb the incudo-malleolar complex or the stapedius. A clean drug delivery/recording catheter, the IntraEar Ecath® (figure 2.1) was then placed in the round window niche. The distal end of the catheter assembly was placed in a pre-positioned retort clamp whilst ensuring that the catheter tubing (and enclosed electrode) was held off the animals' body where it might be subject to movement caused by respiratory excursion. Evidence of any blood around the drill site was cleaned away with a cotton wick and the hole in the bulla plugged with a small cotton ball thus preventing any fresh blood from entering the bulla. Confirmation of correct electrode placement was easily determined using the real-time gross electrophysiological CN signal displayed on the Medelec Sapphire 2A (figure 2.4).

Following surgery, which was usually complete within 20 minutes, the animals' head was gently manoeuvred onto a foam support. This provided comfort as well as allowing access to the animal's snout for oxygen/anaesthetic intervention if required during the experiment. At the end of each experiment the animal was killed by inhalation of 5% Isoflurane followed by exsanguination *in situ*.

2.7. The Intra Ear Ecath®

The Intra Ear Ecath® (Durect Inc, USA) is a triple lumen micro-catheter designed to allow controlled solution delivery to the round window membrane, whilst also allowing electrophysiological recordings (figures 2.1 and 2.2). It is approximately 10cm in length with a total tip diameter of approximately 1mm.

The triple lumen design comprised one inflow, one outflow and one lumen containing an insulated platinum recording electrode, terminating in a fenestrated compressable bulb. This compression was necessary to allow for a tight fit in the bony and inconsistently shaped round window niche of the guinea pig. The inflow lumen allowed the addition and removal of fluid from the round window niche without compromising recordings (figure 2.2).

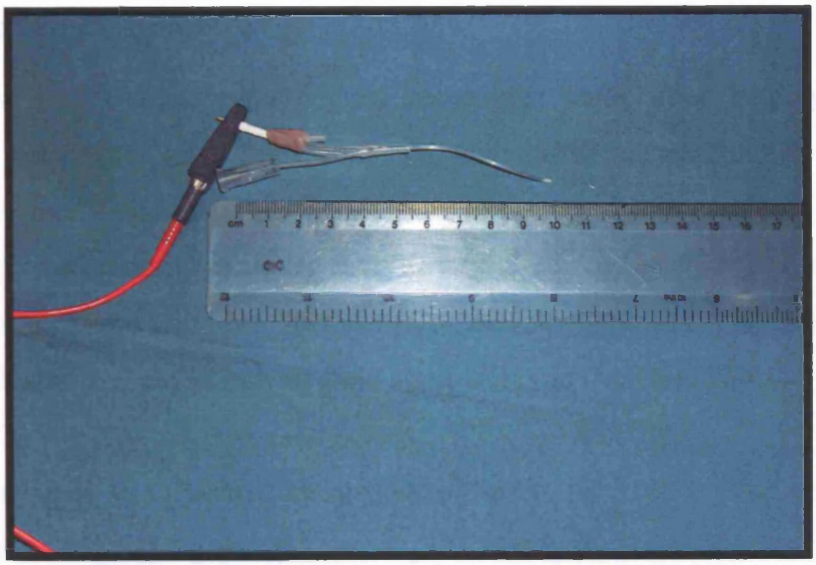


Figure 2.1. The IntraEar Ecath® and active electrode wire connected to the Medelec Sapphire 2A preamplifier.

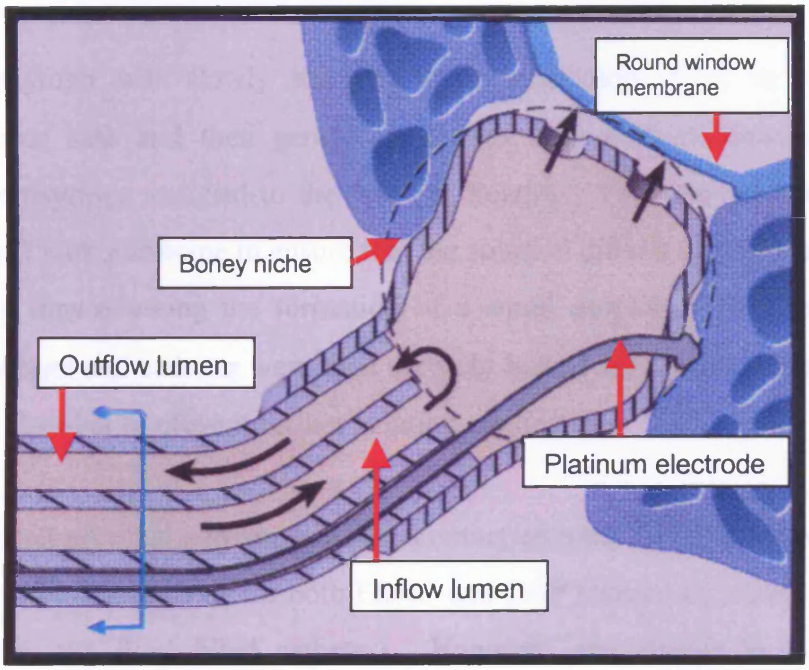


Figure 2.2. A schematic diagram of the IntraEar Ecath® tip and placement within the round window niche.

The outflow lumen terminates in a vent port designed to allow excess drug solution to pass through the catheter, preventing fluid build up and damage to the inner ear. In this thesis, however, the vent port was purposefully blocked off to allow a set amount of artificial perilymph (5µL) to be delivered to the round window ensuring reproducibility across experiments (artificial perilymph preparation is described in section 2.16). The platinum electrode terminated in a small ball allowing optimal contact with the round window membrane or surrounding bone.

2.8. Artificial Perilymph

Following the initial electrophysiological recordings made after surgery, all further recordings in Chapters 4 and 5 were collected whilst artificial perilymph was applied to the round window. This was to ensure stability of the preparation and that there was no unnecessary drift in visually assessed CAP threshold or drop in ESAC power after fluid application to the round window. This also provided the opportunity to assess the use of the IntraEar Ecath® and any effects of fluid application on CAP and ESAC.

Artificial perilymph was prepared according to Puel *et al* (1995). The constituents (in mM) comprised $Na^{+}Cl$ (137) KCl (5) $Ca^{2+}Cl_2$ (2) $MgCl_2$ (1) Hepes (10) and Glucose (11) made up in sterile water for irrigation, adjusted to pH 7.4. Solutions were stored frozen (-20°C) and used fresh on a daily basis.

Artificial perilymph was slowly warmed to approximately 37°C in a temperature controlled water bath and then gently applied to the round window using a 25μL Hamilton Microsyringe attached to the IntraEar Ecath®. The vent port on the catheter was blocked off with plasticine to ensure that the solution did not disperse from the round window niche thus allowing the formation of a small droplet of fluid (approximately 5μL). The syringe and catheter were then securely locked on a small retort stand within the recording chamber to prevent further solution application.

Confirmation that artificial perilymph was in contact with the round window was visually assessed as a drop in amplitude for both ESAC and CAP (caused by changes in electrical resistance along the fluid filled catheter). However, any change in CAP *threshold* resulted in the experiment being stopped. The solution remained in contact with the round window for the duration of the experiment, with the gross integrity of the round window membrane being assessed following removal of the catheter.

2.9. Placement of the acoustic driver

For BAER recordings, the driver employed was a simple earpiece utilised for human neonate recordings. This was carefully placed in the external meatus, after prior examination for debris in the ear canal, by coupling the terminal plastic teat at its opening.

For cochlear recordings, considerably more effort and care was taken when coupling the acoustic driver to the external meatus. This involved the use of a retort clamp holding a

custom made plastic earpiece as shown in figure 2.3. Under microscopic guidance, the coupler was lowered vertically into the ear canal and its position altered using a built in screw gauge until clear visualisation of the tympanic membrane was possible. Removal of debris from the external meatus (eg wax and dead skin) was carried out as needed. Coupling the ear piece holder in position also effectively fixed the position of the animals' head within the foam support.

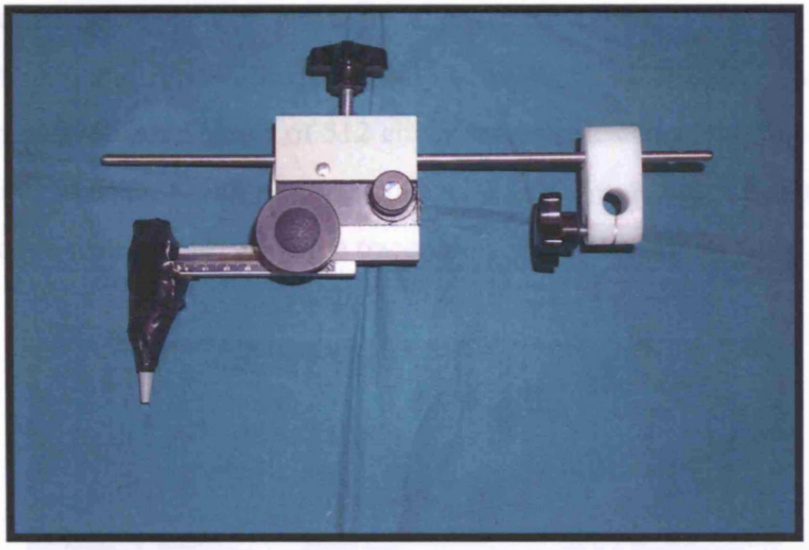


Figure 2.3. The acoustic driver assembly for housing the B and K 4192 microphone.

A Bruel and Kjaer (B&K) 4134 ½" acoustic driver was then placed within the ear piece assembly and initial auditory thresholds at 8, 16, 24 and 30 kHz obtained. If the animal was found to have particularly poor auditory thresholds (below 50 dB attenuation in at least three frequencies) it was rejected.

2.10. Stimulus generation and control

The following sections detail the stimulus generation, and control and exclusion criteria used in this project. Overall, there were three physiological stimulus sources used in these experiments:

- A broadband 100μSec click suitable for eliciting the BAER.
- A short duration tone pip suitable for eliciting a well-defined frequency specific CAP.
- Free field band-pass filtered noise serving as an ototraumatic insult in combination with hypoxia.

2.10.1. Broadband click stimuli - brainstem auditory evoked response (BAER)

The BAER response is generated by the whole auditory pathway (Shaw, 1988) and is typically illustrated in Figure 2.10. A monaural earpiece was placed in the left ear canal, held in place with a small piece of surgical tape attached to the pinna. A broadband 100μSec 'click' stimulus was delivered at a repetition rate of 20 Hz by the Medelec Sapphire 2A, Oxford Instruments (figure 2.4). The frequency spectrum of the click covered up to 10 kHz, but was primarily centred on 4 kHz. The BAER was then amplified x100,000 and filtered predominantly between 10 Hz-5 kHz. The sound click polarity was negative. An average of 512 clicks were presented as the final BAER trace on the Sapphire 2A screen with an input gain of 25μV/div. Adjustments to the display gain were made as necessary to bring the trace into a viewable format.

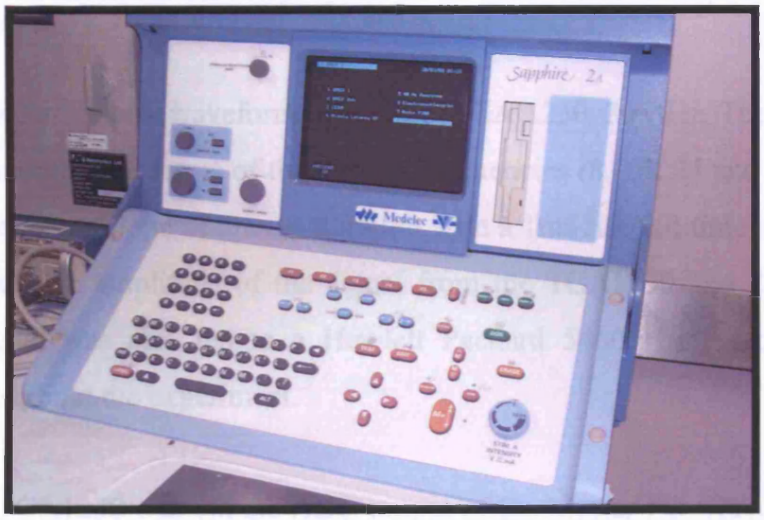


Figure 2.4. The Medelec Sapphire 2A console.

2.10.2. Short duration tone pip - the compound action potential (CAP)

To generate a short, shaped tone pip suitable for eliciting a frequency specific CAP, a computer controlled waveform generator setup was custom designed by Dr PA Jones, Applied Technology, GSK, as shown in Figure 2.5.

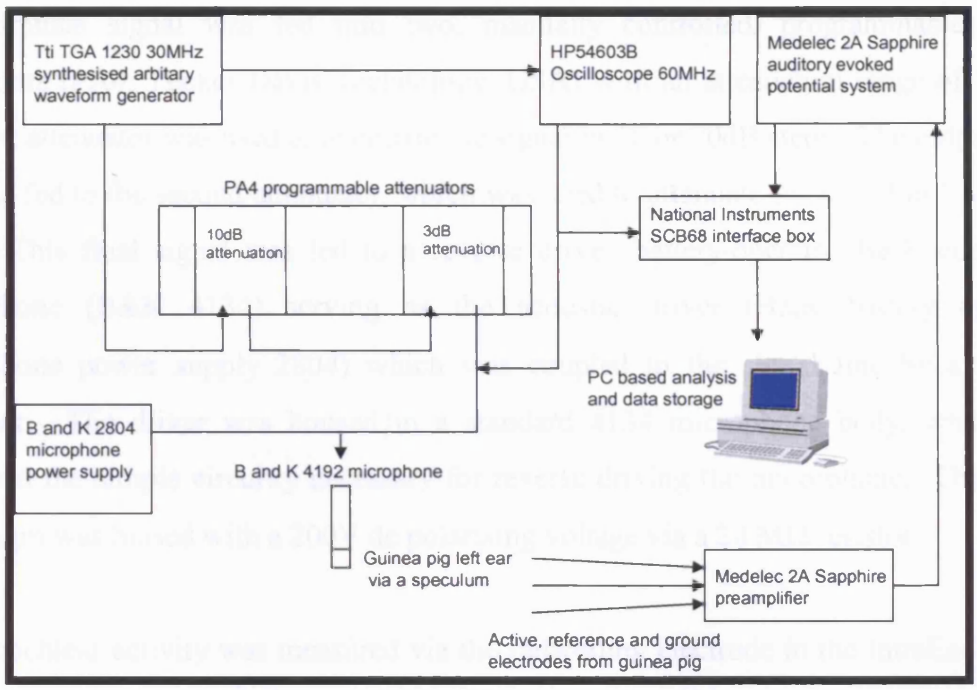


Figure 2.5. The data stimulation and storage system for the measurement of compound action potentials (CAPs).

A Thurlby Thandar arbitary waveform generator (TGA 1230, 30MHz, Tucker Davis, US) was used to generate waveforms of the desired frequencies (8, 16, 24 and 30 kHz) which comprised a cosine envelope of 5ms in duration with a 1ms rise/fall time and a plateau of 3ms. The maximum amplitude of the signal from the TGA1230 was 20Volts peak to peak. The signal was also fed to a Hewlett Packard 54600 oscilloscope for signal monitoring throughout the experiment.

Control of the TGA1230 was via the ADC output of the Master PC (Kayak XA, Hewlett Packard running on NT4.0) allowing control of the software directly from the keyboard. The onset of stimulus generation in turn, triggered capture and averaging of the CAP response by the PC. The rate of stimulus was set at 7 Hz, with repetition of stimuli for averaging being dependent on the stimulus intensity. Normally at 0dB attenuation, averaging of between 20 to 50 CAP responses gave excellent resolution of the digitised trace. As stimulus intensity progressively decreased, averaging was increased up to about x100 to increase resolution of the CAP response.

The resultant CAP signal was pre-amplified, as for the BAER, via the Sapphire 2A preamplifier and this analogue signal was then fed via the ADC input of the ADC sampler at a rate of 16 kHz. The CAP signal was then displayed on the PC with sensitivity (Volts/division) and time base (ms/division) set manually.

The stimulus signal was fed into two, manually controlled, programmable digital attenuators (PA4, Tucker Davis Technology, USA) with an attenuation range of 100dB. The first attenuator was used to attenuate the signal in 10 or 20dB steps. The output from this was fed to the second attenuator, which was used to attenuate the signal in 1 to 3, dB steps. This final signal was fed to a reverse driven battery-operated B&K condenser microphone (B&K 4134) serving as the acoustic driver (B&K battery operated microphone power supply 2804) which was coupled to the signal line by a 0.01μF capacitor. The driver was housed in a standard 4134 microphone body, which also contained the simple circuitry necessary for reverse driving the microphone. The driver diaphragm was biased with a 200V dc polarising voltage via a 20 MΩ resistor.

Gross cochlear activity was measured via the indwelling electrode in the IntraEar Ecath® that recorded the electrical signal via the fluid interface over the round window/promontory.

2.10.3. Free-field band pass filtered noise generation as an acute cochlear insult alone and in combination with hypoxia

A schematic representation of this system is shown in figure 2.6. Free-field band pass filtered noise was used (average 100 dB SPL) both alone and in combination with hypoxia as cochlear insults. An in-house white noise generator and amplifier were connected to a Sound Lab graphic equaliser DEQ 31 x 1 that acted to band-pass filter the white noise between about 5 and 20 kHz (figure 2.7). Signals above this were attenuated at 6 dB/octave. Frequencies below 5 kHz were attenuated by at least 24 dB.

This modulated band pass filtered signal was split by a crossover octave circuit between three loud speakers built into the lid of the perspex experimental chamber (approximately 12" in height). Low frequencies were handled by a 4.5" high power driver (RS model 249-902) medium by a 4" mid range driver (RS model 250-211) and high frequencies by a power line tweeter.

Prior to noise exposure the B&K driver was removed from the *in situ* acoustic coupler leaving the animals' ear canal clearly open facing the driving loud speakers. This secure arrangement ensured that each animal was subject to, as far as possible, an equivalent exposure level of noise insult. Noise calibration was carried out with the calibration microphone coupled to the end of the earmould to reflect the SPL level at the guinea pigs

ear as close as possible. The average noise level measured at this location was typically about 100 dB SPL (figure 2.8).

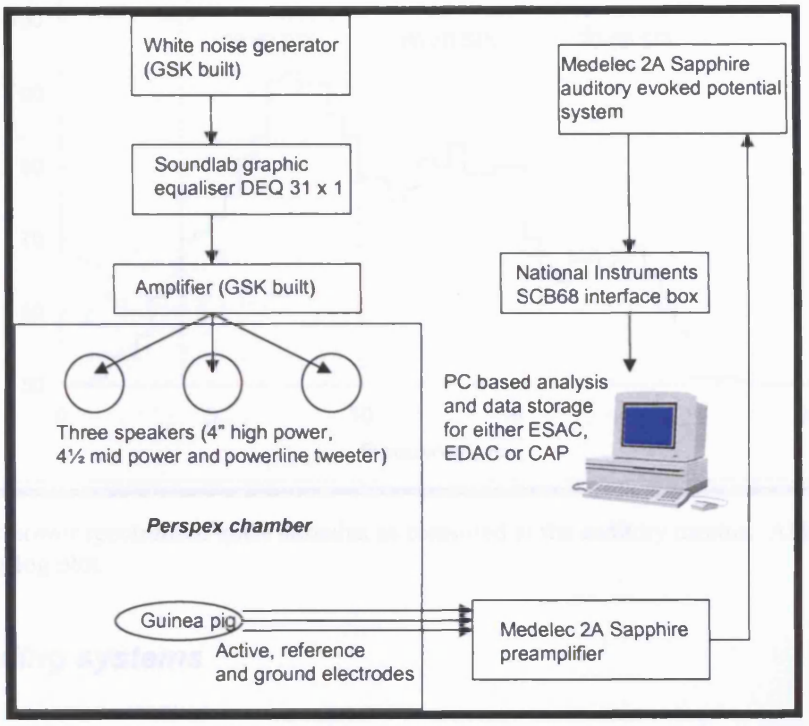


Figure 2.6. Schematic diagram of the white noise generation and data collection/ storage system.

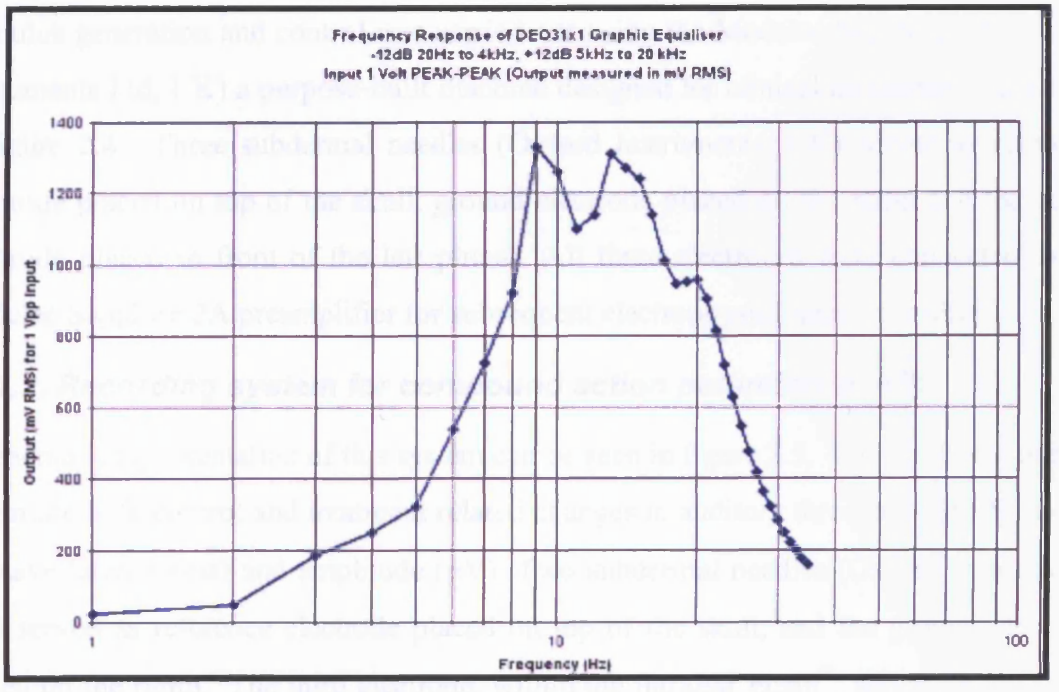


Figure 2.7. Electrical frequency response plot taken from the Sound Lab graphic equaliser showing peak energy over the frequency range 5 to 20 kHz.

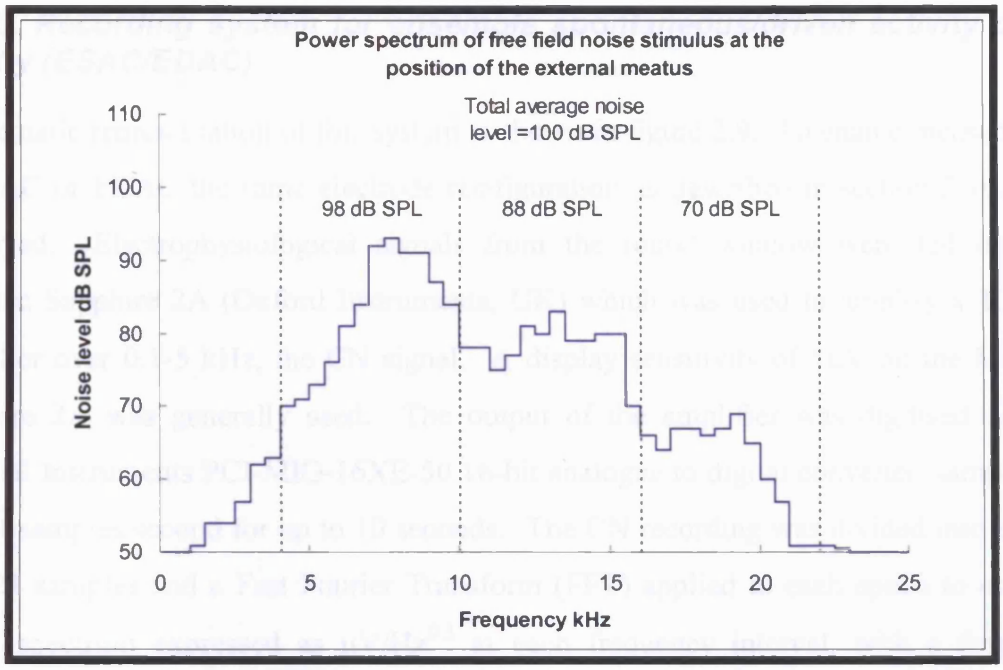


Figure 2.8. The power spectrum of noise stimulus as measured at the auditory meatus. Although not shown here, this is a log-log plot.

2.11. Recording systems

2.11.1. Recording system for the brainstem auditory evoked response (BAER)

Stimulus generation and control was carried out using the Medelec Sapphire 2A (Oxford instruments Ltd, UK) a purpose-built machine designed for clinical audiometry, as shown in figure 2.4. Three subdermal needles (Oxford Instruments, UK) served as reference electrode placed on top of the skull, ground electrode placed on the rump and the active electrode placed in front of the left pinna. All three electrodes were connected to the Medelec Sapphire 2A preamplifier for subsequent electrophysiological recording.

2.11.2. Recording system for compound action potentials (CAP)

A schematic representation of this system can be seen in figure 2.5. The CAP was used to determine both control and treatment related changes in auditory thresholds (dB SPL) and N_1 wave latency (ms) and amplitude (μV). Two subdermal needles (Oxford Instruments, UK) served as reference electrode placed on top of the skull, and the ground electrode placed on the rump. The third electrode, within the IntraEar Ecath®, served as the active electrode. All three electrodes were connected to the Medelec Sapphire 2A preamplifier for subsequent electrophysiological recording. All data was collected, stored and subsequently analysed using PC based software running on Windows NT4.0.

2.11.3. Recording system for ensemble spontaneous/driven activity of CN activity (ESAC/EDAC)

A schematic representation of this system is shown in figure 2.9. To enable measurement of ESAC or EDAC the same electrode configuration as described in section 2.10.2 was employed. Electrophysiological signals from the round window were fed into the Medelec Sapphire 2A (Oxford Instruments, UK) which was used to amplify x 100,000 and filter over 0.1-5 kHz, the CN signal. A display sensitivity of 5μV on the Medelec Sapphire 2A was generally used. The output of the amplifier was digitised using a National Instruments PCI-MIO-16XE-50 16-bit analogue to digital converter, sampling at 16,384 samples/second for up to 10 seconds. The CN recording was divided into epochs of 1024 samples and a Fast Fourier Transform (FFT) applied to each epoch to obtain a power spectrum expressed as $\mu V/Hz^{0.5}$ at each frequency interval, with a frequency resolution of 16 Hz. The power spectra were averaged to obtain the average spectrum of CN activity. The total power was calculated by summing the power at each frequency interval over the range of the spectrum (see figure 2.12 for an example of an ESAC recording).

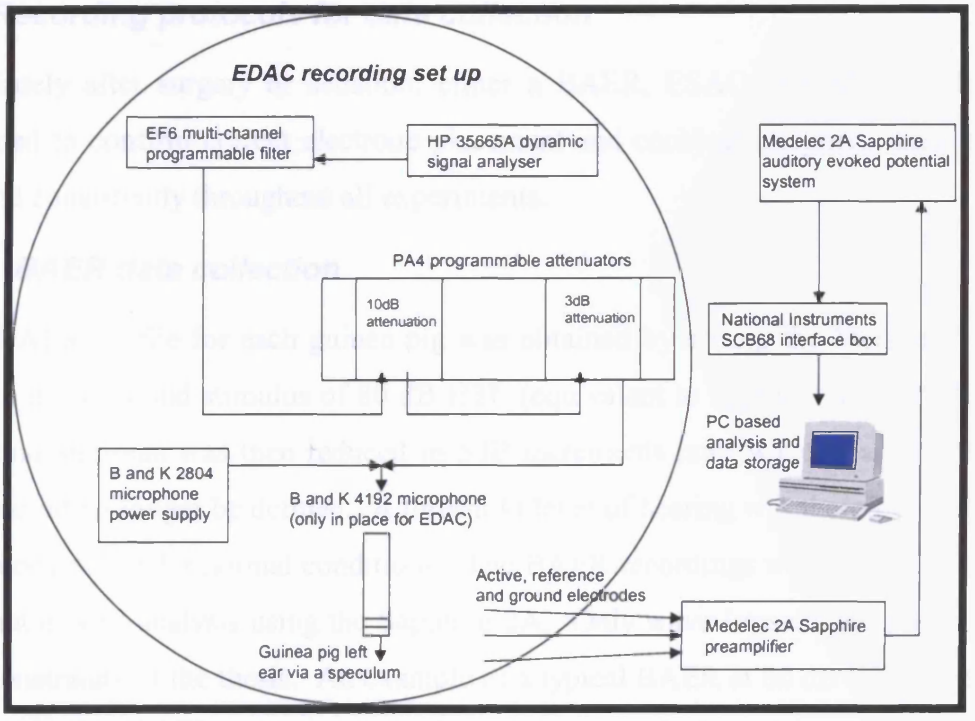


Figure 2.9. The data stimulation and storage system for the measurement of ensemble spontaneous and driven CN activity (ESAC/EDAC).

The difference in power between ESAC spectra was calculated in dB (dB = 10 log [P1/P2]) where P1 was the pretreatment recording and P2 was a recording taken during time controlled experiments. Pre-treatment ESAC values were always used as a baseline

measurement unless otherwise stated. Different frequency domains of the ESAC power spectrum were studied including 0.5 to 1.5, 1 to 3, 0.5 to 8 and 0.1 to 8 kHz. Only results from the 0.5 to 1.5 kHz power region are presented in these experiments, as these represent the dominant frequency Na^+ channel activation/inactivation kinetics (Dolan *et al,* 1990). All ESAC/EDAC data was collected, stored and subsequently analysed using PC based software running on Windows NT4.0.

2.11.3.1. The fast Fourier transformation (FFT)

In studying ESAC or EDAC it is important to briefly re-emphasise the Fast Fourier transformation or FFT as previously described in Chapter, section 1.5. In essence, the Fourier transform, decomposes or separates a spectral waveform or function such as CN activity into sinusoids of different frequency which sum to the original waveform; in this case ESAC/EDAC. It identifies or distinguishes the different frequency sinusoids and their respective amplitudes that can then be analysed. Thus an ESAC recording over a few seconds can be broken down to form the characteristic spectrum of nerve activity as shown by Cazals (1998). Figure 2.12 shows an example of ESAC collected in this experiment.

2.12. Recording protocols for data collection

Immediately after surgery or sedation, either a BAER, ESAC or CAP recording was performed to confirm correct electrode placement and cochlear integrity. This protocol was used consistently throughout all experiments.

2.12.1. BAER data collection

A full BAER profile for each guinea pig was obtained by setting the Medelec Sapphire 2A at an initial sound stimulus of 80 dB HTL (equivalent to approximately 95 dB SPL). The sound stimulus was then reduced in 5dB increments until waves I and/or V of the BAER could no longer be defined. A threshold level of hearing was therefore defined for each guinea pig under normal conditions. The BAER recordings were stored on disk for future automated analysis using the Sapphire 2A. Only wave I results are presented due to the constraints of the thesis. An example of a typical BAER at 80 dB HTL is shown in figure 2.10. The BAER was assessed at 15 minute intervals throughout the experiment in each group. All recordings were made in the perspex chamber and any noise stimulus, ie white noise exposure, was turned off.

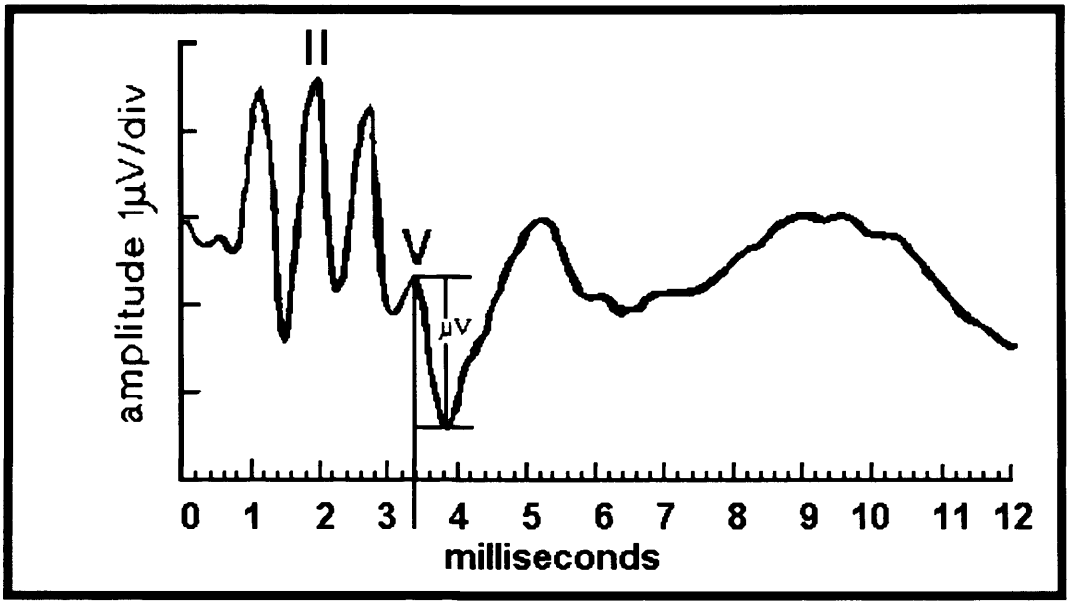


Figure 2.10. A typical guinea pig BAER collected at 80dB HTL. Waves II and V are highlighted to show wave positioning. Wave V also shows the criteria used for measuring wave amplitude and latency, although in this thesis Wave I amplitude and latency were actually measured.

2.12.2. CAP data collection

An example of a CAP data capture is shown in figure 2.11. The CAP waveform displayed was refreshed with every averaged signal and a clear response became visible within seconds. The initial maximal stimuli presented at 8, 16, 24 and 30 kHz are defined in section 2.12.2 and presented in table 2.1. Stepwise attenuation was then performed until a visually assessed threshold level was established, defined as a non-measurable N_1 waveform. Once averaging was complete, the resulting CAP 'display' was saved as a unique data file for offline analysis using in-house designed software. After the application of artificial perilymph, the order of CAP recording was always 30, 24, 16 then 8 kHz. This proved to be the most reliable method for capturing rapidly changing thresholds at the basal or high frequency end of the cochlea due to the location of the recording electrode at the round window. Recordings were made at predetermined times throughout each experiment thus, each animal had a complement of files corresponding to the response found to each attenuation at each frequency tested.

Along with threshold estimation at each frequency, stimulus levels of 0, 20, 40 and 60 dB SPL attenuation were delivered to generate data at different points of the CAP growth function. For these, the amplitude and latency of the N_1 peak were measured offline and stored on a Microsoft Excel spreadsheet for manipulation and plotting.

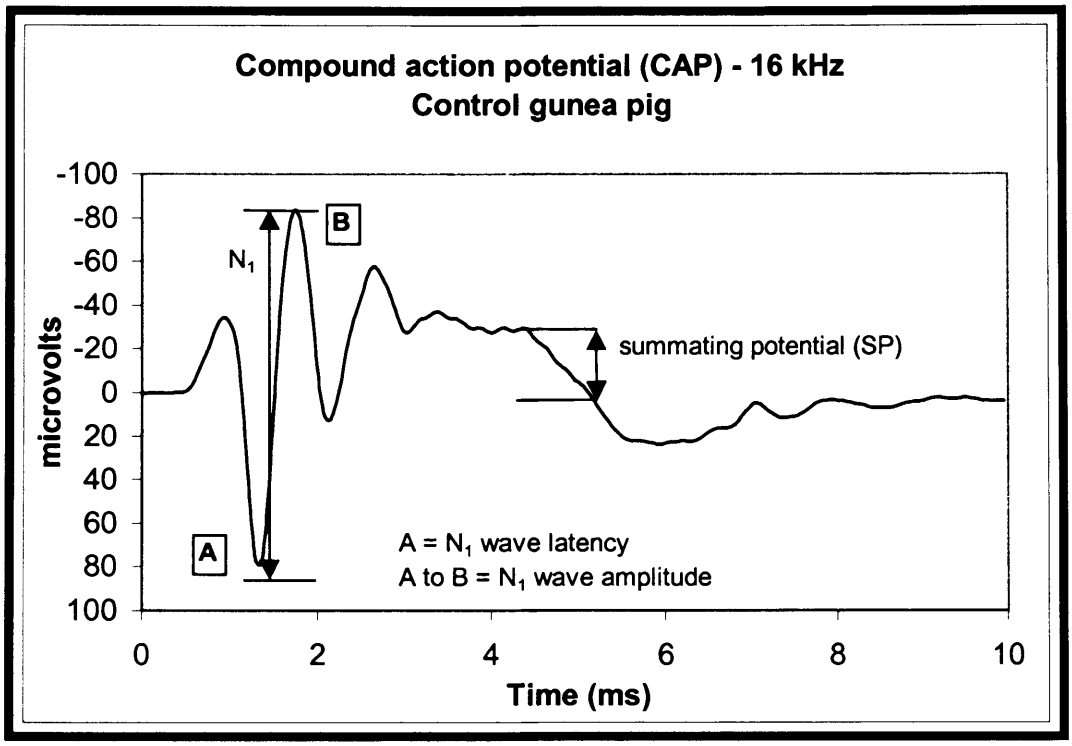


Figure 2.11. Control guinea pig CAP data capture (taken after stimulus at 80dB, 16 kHz). N_1 latency and amplitude were measured along with threshold. Amplitude measurement was from the early positive deflection prior to the furthest negative deflection marked 'N_1' on the diagram. The summating potential (SP) is shown for interest only.

2.12.3. ESAC/EDAC data collection

An example of the ESAC data capture following FFT analysis is shown in figure 2.12. Recordings of usually 10 seconds in length, in the absence of signal delivery, were made at predetermined times during each experiment, as well as during noise exposure (recordings were then termed EDAC). Respiratory excursions, hiccoughs or other animal movement artifacts would result in that specific ESAC recording being rejected and a subsequent period of data capture would be repeated. Usually, at least two ESAC recordings were performed at separate timepoints prior to any treatment or noise exposure. This ensured that the animal was stable and that there was no drift in ESAC power. The characteristic shape of the ESAC plot was not dependent on the length of the recording so, in less stable preparations, shorter time recordings were feasible.

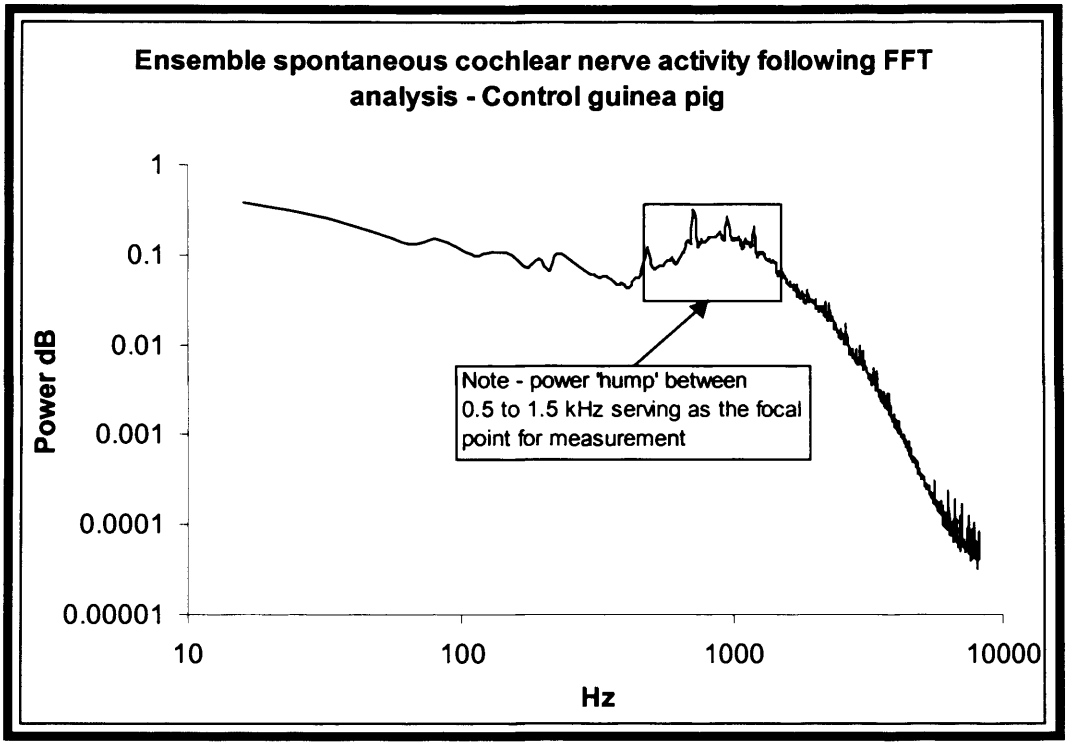


Figure 2.12. Control ensemble spontaneous CN activity (ESAC) following FFT analysis. The power 'hump' between 500 and 1500 HZ formed the basis of interest in this thesis. The artefactual spiking observed below approximately 300 HZ was occasionally seen due to electrical interference and muscle movement. Some higher peaks may have also represented discrete physiological correlates of OAEs.

The ESAC/EDAC data values of each 16 Hz data bin were transferred and stored to be analysed offline using software written by Dr PA Jones, GSK. This software was used to calculate the power over the 500 Hz–1.5 kHz bandwidth. These values were then entered into a Microsoft Excel spreadsheet for manipulation and plotting.

2.13. Calibration of the sound systems

2.13.1. Medelec Sapphire 2A calibration

This system was pre-calibrated by Medelec, UK. For the purposes of this study, a stimulus level of 0dB HTL was calibrated as being equivalent to 15dB SPL, according to the manufacturer's specification (Oxford Instruments UK).

2.13.2. Calibration of closed field stimulus delivery used for cochlear recordings

The B&K 4134/4192 driver was coupled via a reciprocity closed calibration chamber B&K Type 4142 (a B&K 4134 microphone was used as the reciprocal calibrator). The B&K 4134 driver was then continuously driven by the TGA1230 at 20 volts peak to peak with the 200V bias voltage provided by the B&K 2804 power supply which also powered the 4134 microphone. A secondary output from the B&K 2804 was fed to the Hewlett Packard 35665A signal analyser, which gave a graphic display of signal intensity in dB

SPL. The driver was calibrated over 1 to 30 kHz and found to operate within the B&K specification. Table 2.1 shows the maximal output values, in terms of dB SPL, obtained at 8, 16, 24 and 30 kHz respectively.

The source of acoustic calibration was provided by a 1 kHz tone generator that delivered a 112 dB SPL tone. The calibration of this device was carried out by the National Physics Laboratory, Teddington, UK. The output of the B&K 2804 was also visually checked on an oscilloscope, which was used to inspect shaping, and symmetry of the stimulus waveforms.

Table 2.1. Maximal driver output (dB SPL) after closed field stimulus at 8, 16, 24 and 30 kHz.

Frequency (kHz)	Voltage (p-p) mV	dB SPL
8	35	92
16	36	92
24	10	82.5
30	10	84

2.14. Induction of Hypoxia

The purpose built perspex recording chamber also served as the hypoxia chamber. The normal atmosphere within the chamber was controlled by a permanent supply of compressed air that was maintained at a flow rate of approximately 5L/minute. On initiation of hypoxia, the compressed air supply was reduced and the nose cone (present in case of supplemental doses of gaseous anaesthesia) was moved away from the animals' head. The various holes in the chamber for recording wires etc. were also sealed. Nitrogen (BOC Cylinder) was then flushed into the chamber and the intra chamber oxygen concentration was monitored using a battery operated oxygen meter (either the Oxywarn 100R or the Neotronics oxygen monitor, New Jersey USA) with a sensor constantly in place within the chamber. The nitrogen flow rate was controlled using a flow moderator valve to achieve a steady state of either 18, 16, 14 or 12% hypoxia +/- 0.5%. Air within the chamber was regularly sampled and the O_2 concentration within the chamber was instantly displayed on the LED screen of the O_2 meter.

This system allowed a rapid and stable hypoxic atmosphere to be attained within 30 to 40 seconds of starting delivery of the gas mix. Pulse oximetry confirmed the efficacy of the

procedure. At the end of the experiment, the nitrogen was discontinued and normal air supply was resumed. This rapidly led to a return in pulse oximetry values of over 97%.

2.15. Induction of Noise

As a cochlear insult by itself, free field band pass filtered noise was delivered following adequate control BAER, CAP or ESAC recordings. In chapter 3, noise exposure was for up to 60 minutes but, for Chapters 4 and 5, only 15 minutes noise exposure was employed. No modification of the calibrated noise source/signal was required during any experiment. When both noise and hypoxia were given in combination, delivery of both was initiated and ended at the same time within the defined exposure periods as stated in the relevant chapters. A full set of recordings was then performed post noise exposure to complete the experiment. Full details are presented in Chapters 3, 4, and 5.

2.16. Collection of perilymph and blood samples

In Chapter 5, both blood and perilymph samples were collected after an overdose of pentobarbitone. Terminal blood samples for plasma (up to 5mL) were taken via cardiac puncture using a needle and syringe into heparinised pots, which were subsequently mixed and centrifuged at 3000g. for 15 minutes. Perilymph sampling was performed following decapitation. The bulla was rapidly removed and thoroughly cleaned to remove all tissue debris and external fluids. Using a microscope, a glass micro-pipette attached to a short length of pp10 tubing and a Hamilton 25μL microsyringe was used to penetrate the round window membrane. A small retort stand was also employed to provide stability to the sampling procedure. This system generated only a small negative pressure allowing gentle aspiration of perilymph. All samples were stored frozen in Eppendorf tubes at approximately –20°C for subsequent analysis.

2.17. The assay of plasma and perilymph for lamotrigine (LTG)

Levels of total LTG in both plasma and perilymph were determined using a protein precipitation extraction procedure using high performance liquid chromatography (HPLC, Waters 600 Multisolvent Delivery System) with ultraviolet detection at a wavelength of 302nm (Applied Biosystems Programmable UV absorbance detector).

The LTG retention time was 5.4 minutes and quantification was carried out using area under the peak. To each standard, 1mL of acetonitrile was added and 500μL of plasma from each sample. Samples were vortexed and centrifuged for 15 minutes at 13000g. to sediment the protein. The supernatant was removed and evaporated to dryness under

nitrogen at 37°C. Samples were resuspended in 300μL of mobile phase (75% 0.05M ammonium acetate pH5: 25% Acetonitrile) and an injection of 150μL made. An aliquot of perilymph was taken and the volume made up to 170μL with the addition of the mobile phase (75% 0.05M ammonium acetate pH 5: 25% Acetonitrile) and an injection of 150μL made. The data capture system used was Multichrom.

2.18. Statistical analysis

Data were arranged in Excel spreadsheets and subsequently sorted according to time and treatment groups. They were then transferred to MINITAB (Minitab Inc., release no.13.31) and analysed using one way repeated measures ANOVA for *within group* time/treatment effects. A significance value of 0.05 was adopted for the F-test. If this level was reached then post hoc testing was carried out using Dunnett's *post hoc* test. This test returned the 95% confidence interval between the control mean and all other time point treatment means, and was used as the basis for determining the significance of any effect.

For *across group* comparisons, one way ANOVA of normalised data was carried out and again a significance value of 0.05 was adopted for the F-test. If this level was reached, then *post hoc* testing was carried out using the more conservative Tukey's *post hoc* test. This test returned the 95% confidence interval between any given pair of control or treatment means. This was used as the basis for determining the significance of any effect.

Chapter 3. The effects of hypoxia, noise and noise/hypoxia combination on the BAER

3.1. The development of a stable noise/hypoxia (NH) model of excitotoxicity in the guinea pig

This chapter covers the preliminary work necessary for the development of a stable NH model of excitotoxicity in the guinea pig using the brainstem auditory evoked response (BAER). This model was based on the hypothesis that moderate acute bandpass noise of approximately 100 dB SPL in combination with stable marginal atmospheric hypoxia (12% oxygen) would result in increased post synaptic glutamate concentrations. This would be due to a compromised glutamate re-uptake within the cochlea as a consequence of hypoxia (Mayer *et al* 1987, Stys *et al* 1992, Kiedrowski *et al* 1994a-b, Hyodo *et al* 2001). The resultant outcome would be that lower levels of noise stimuli would be required to induce excitotoxic damage in the auditory system.

In developing this acute NH model of cochlear insult, it was necessary to validate the BAER measurement in the guinea pig, as this had not previously been performed within GSK. In developing this model, the aim was to empirically establish the lowest level of hypoxia that would not compromise the general physiological stability of the preparation. This involved the measurement of electrocardiography (ECG), basal temperature, spontaneous ventilation and monitoring the level of involuntary movement by the animal.

The effects of systemic functional deficit on the cochlea have been reported by a number of workers. For example, relatively modest reduction in mean arterial blood pressure has been reported to result in decreased cochlear sensitivity and an increased refractory period in the CN (Evans 1972, 1974, Mulheran and Evans, 1988). Reductions in basal temperature have also been shown to adversely affect cochlear function (Watanabe *et al*, 2001).

It was also necessary to establish the optimal acute anaesthesia regime in the guinea pig. The aim here was to achieve adequate anaesthetic depth, whilst avoiding disturbance to systemic physiology and peripheral neural function. This was especially desirable with the use of hypoxia. Without sufficient anaesthetic depth, the likelihood of involuntary movements in the guinea pig would increase, leading to difficulties in obtaining stable BAER due to the swamping of the small (μV) potentials by the much larger (mVs)

myogenic potentials. This also applied to capture of ESAC data discussed in Chapters 4 and 5.

In establishing that these measurements remained stable, there would be a greater level of confidence that any effects on BAER were due to the direct effects of hypoxia on the cochlea, rather than due to secondary systemic effects. Once an optimal level of hypoxia was established this could then be utilised in combination with noise exposure to provide what was expected to be a synergistic challenge to the cochlea.

3.2. Rationale for using a minimum of 12% hypoxia in combination with noise as an acute cochlear insult

Consideration of an optimal hypoxia level for use in this study was informed by reviewing the relevant literature (see Chapter 4, section 4.8). The use of hypoxia levels at or below 10% in an acute animal model was not deemed to be suitable in this study. This was because of the likelihood of the confounding effects on systemic physiology mentioned above (Attias *et al*, 1990). It was apparent from the literature that no prior BAER study had considered utilizing a more marginal level of hypoxia of approximately 12% oxygen. At this level of oxygenation, inspection of the standard oxyhaemoglobin dissociation curve shows that the corresponding alveolar level partial pressure is approximately 58mmHg. This partial pressure would be associated with a value of approximately 85% oxyhaemoglobin saturation (figure 3.1) which does not appear to be associated with acute disturbance to the cardiovascular system at rest. For reference, the usual oxygen saturation in arteries is 97%, which equates to an alveolar partial pressure value of 95mmHg (Guyton and Hall, 1996).

As a consequence, it was decided to first investigate the stability of acute atmospheric hypoxia down to 12% in the guinea pig. If this proved successful, then the model could be developed further by combining this level of hypoxia with moderate noise as an acute model of ototrauma. In particular, this would result in overwhelming the acute glutamate re-uptake response in the cochlea, that in turn would be expected to lead to an acceleration or synergising of the excitotoxic damage caused by the effects of noise.

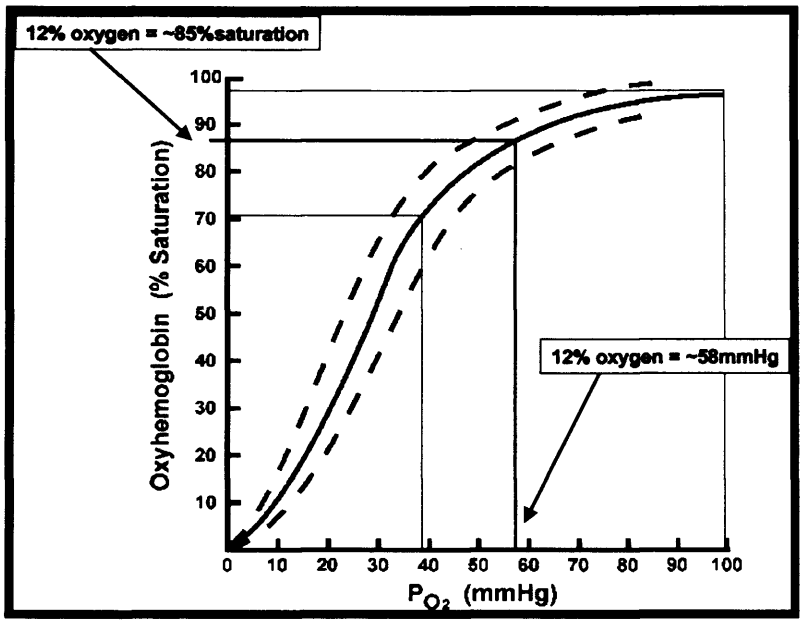


Figure 3.1. The oxygen dissociation curve of alveolar/arterial blood.

3.3. Experimental methods

The results from four sets of experiments are presented in this chapter. The aims of these preliminary studies were to fulfil the following criteria using measurement of the BAER as a suitable endpoint:

- To investigate the best neuroleptanaesthesia regimen for repeated monitoring of BAER for up to at least 2 hours, with minimal interference to electrophysiological measurements or the need for respiratory intervention. It was anticipated that the definitive sedative regimen would then be used for the remainder of the experiments in this thesis.

- To perform studies investigating the effects of moderately reduced oxygen, noise or a combination of the two for one hour on the BAER (followed by one hour recovery) in an attempt to establish a robust model for future studies of peripheral tinnitus and protection against noise induced hearing loss (NIHL).

Animal supply, husbandry, diet and drinking water, and full methodology for the BAER and ECG are given in the relevant sections in Chapter 2, but a brief description is provided for each experiment below.

3.3.1. Experiment 1 - Validation of BAER recording in the guinea pig

A preliminary experiment was conducted to provide background BAER data whilst ensuring that the animals would remain sedated for at least one hour. A total of 12 guinea pigs were used following sedation Regimen 1, as described in Chapter 2, section 2.4.

3.3.2. Experiment 2 - The effects of 21 to 12% oxygen on the BAER over 60 minutes followed by recovery

This experiment was designed to assess the effect of 21 to 12% oxygen on the BAER over at least 60 minutes. In the first instance, a total of 12 guinea pigs were randomly allocated by hand to four groups as shown in Table 3.1. Animals were again sedated using sedation regimen 1 and then exposed to either 12, 14, 16, 18 or 21% oxygen for one hour followed by recovery in normal atmospheric conditions for up to one hour. Recordings of BAER were also taken at 15 minute intervals during the recovery period. Five additional guinea pigs (Group 6) were also studied at 12% O_2 for one hour using sedation regimen 2 (Chapter 2, section 2.4).

Table 3.1. Experiment 2 - Animal allocation and oxygen (O_2) exposure

Group Number	Approximate level of atmospheric O_2 (%) **	Number of animals
1* control	21	4
2	18	3
3	16	3
4	14	3
5	12	3
6	12	5

* Animals that served as control (21% oxygen) were reused throughout the other groups after at least 3 days between sedation periods (total no. of animals used was therefore 17).

** All levels of oxygenation were maintained within +/- 0.5% of nominal.

3.3.3. Experiment 3 - The effects of 12% oxygen, noise or noise/12% oxygen (NH) on the BAER, pulse rate and ECG

Further BAER work was performed using sedation regimen 3 (Chapter 2, section 2.4). This study comprised four treatment groups of 35 animals as shown in Table 3.2. Animals were either exposed to normal air for up to 2 hours (control) or they were exposed to 60 minutes of band pass noise at approximately 100 dB SPL, 12% oxygen or a combination of both noise and hypoxia (NH) followed by recovery in normal air

(maximum of 1 hour for each). Both ECG and pulse rates were also monitored, as described in Chapter 2.

Table 3.2. Experiment 4 - Study Design

Group Number	Treatment	Number of animals
1	Control (atmospheric oxygen)	13
2	Hypoxia (12% oxygen)	10
3	Noise (100 dB)	6
4	Hypoxia (12% oxygen) and Noise (100 dB) –NH	6

3.4. Results

3.4.1. Experiment 1 - Preliminary BAER studies in the sedated guinea pig; selection of an optimal acute neuroleptanaesthesia regime

Preliminary studies provided experience in the successful recording of stable BAER data under normoxia. It was noticed that some animals showed signs of involuntary movements, manifest as ear flicking, limb twitching, occasional mild body tremor or chewing movements. These movements affected recording stability, especially at lower stimulus levels when the evoked response was small, ie. <1 μVolts.

Following this, and with veterinary advice, a modification to the sedative regime was performed. Initially, a combination of Hypnorm®/Hypnovel® was used. It has been shown that Hypnovel® (Midazolam) on its own does not induce anaesthesia in rodents even at doses as high as 30mg/kg, but it has been shown to potentiate the effects of fentanyl (Sublimaze®) and droperidol (Pieri, 1981). Results obtained by Flecknell (1984) showed that Hypnorm® (fentanyl/fluanisone) and Hypnovel® combined produced neuroleptanalgesia with good skeletal muscle relaxation in mice, rats, gerbils, hamster, guinea pigs and rabbits.

However, involuntary movements were still observed in the preparations so a third sedative regime was used. This regime medetomidine (Domitor®) and Fentanyl (Sublimaze®) combined showed that the degree of involuntary movement was much lower than with the previous two regimes. This dosage was repeated every 15 minutes for

optimal neuroleptanaethesia. Consequently, Domitor/Sublimaze® was then adopted for use throughout the rest of the study.

3.4.2. Experiment 2 - The effects of hypoxia on the BAER over 1 hour followed by 1 hour recovery

Pilot studies showed that stable BAER recordings could be made under atmospheric hypoxia down to 12%. Recordings were made for 1 hour using either atmospheric oxygen (21%), 18, 16 14 or 12 % oxygen followed by recovery under normoxia for up to one hour. As successful BAER recordings were made down to 12% in five animals this level was then adopted for use in the further development of this model.

3.4.3. Experiment 3 - The effects of 12% hypoxia, noise or noise/12% oxygen (NH) on the BAER, pulse rate and ECG

It was considered to be particularly important for this study to establish that general physiological stability was not affected by 12% hypoxia. The effects on pulse rate; appearance of the ECG, basal temperature and spontaneous respiration are discussed in more detail.

3.4.3.1. The effects of 12% hypoxia on pulse rate

The effects of 12% hypoxia for 60 minutes on pulse rate are shown in figure 3.2. This plot shows that the control values were very stable over the two hour recording period. During 12% hypoxia, a non-significant decrease was seen in the mean rate amounting to a percentage drop of about 17% at 30 minutes (from approximately 230bpm to 200bpm). This rapidly recovered to near control values after reverting to normoxia. The resting rate for these guinea pigs was relatively low compared to the normal physiological range of approximately 180-350 beats/minute (Poole, 1999) but was within the quoted range.

3.4.3.2. The effects of 12% hypoxia on ECG

Figure 3.3 shows that 12% hypoxia resulted in short lived changes in the ECG 'PQRS' complex. Gross ECG changes comprised a pronounced increase in ST segment depression within 10 to 15 minutes after exposure (figure 3.3) and a brief increase in S wave amplitude. This came back to normal during the remainder of the period. This is recognised as a normal response to moderate hypoxia and again suggests cardiac function was not markedly compromised by the 60 minute hypoxia period (Nuttall and Lawrence, 1980).

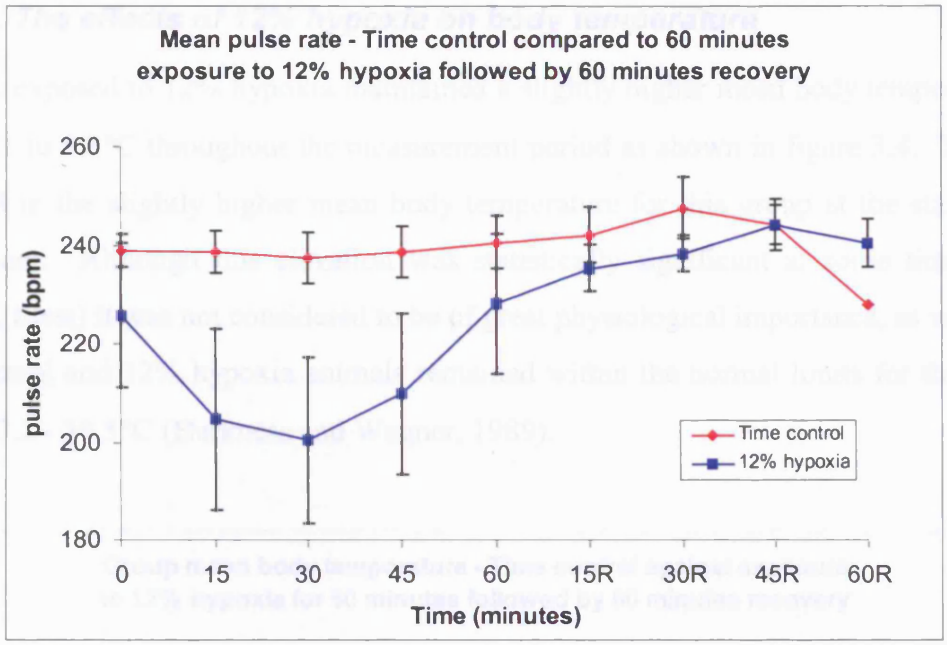


Figure 3.2. The effect of 12% hypoxia on mean heart rate (beats per minute) compared to control heart rate. Whilst decreases in the mean pulse rate were seen during 12 % hypoxia these were not significant. The rapid return to normal mean pulse rate suggests that this level of hypoxia did not result in any serious functional deficit in heart rate. (R – denotes during recovery period).

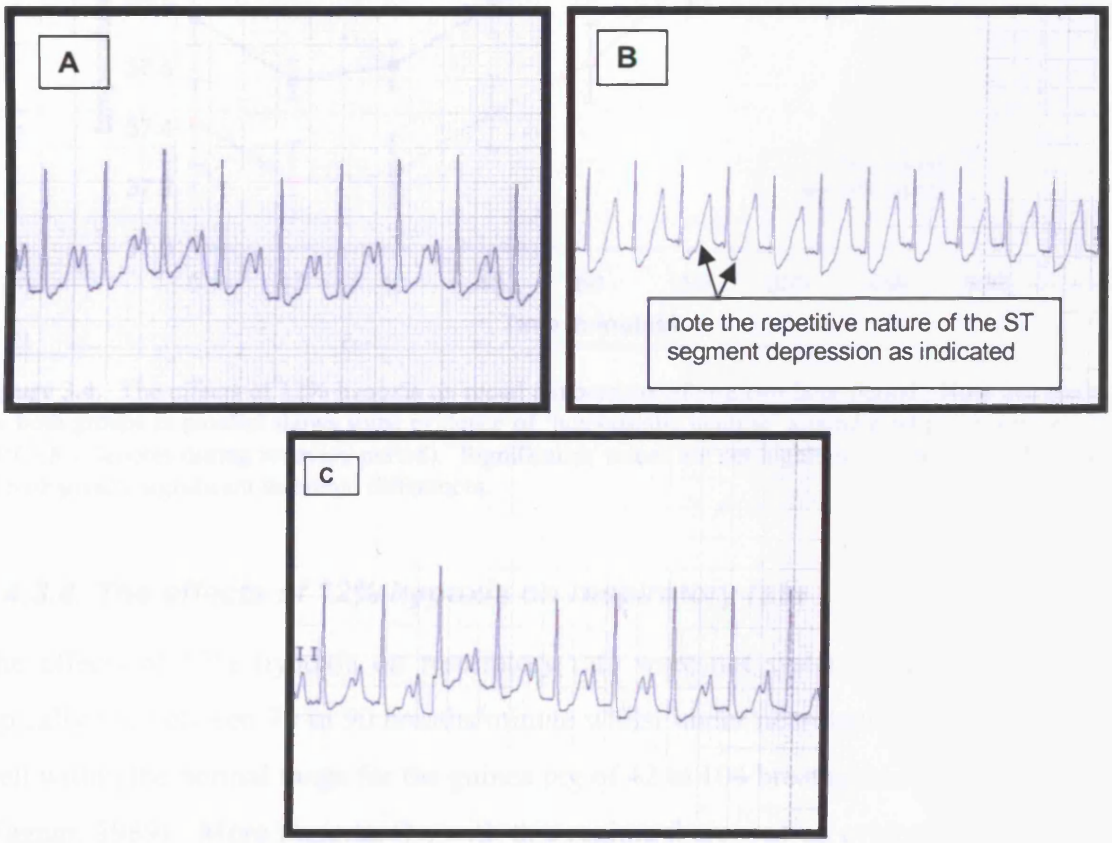


Figure 3.3. An ECG taken (A) before 12% hypoxia (B) 10 minutes exposure to 12% hypoxia showing marked ST segment depression and (C) after 60 minutes exposure to 12% hypoxia showing full recovery.

3.4.3.3. The effects of 12% hypoxia on body temperature

Animals exposed to 12% hypoxia maintained a slightly higher mean body temperature of about 0.1 to 0.3°C throughout the measurement period as shown in figure 3.4. This was reflected in the slightly higher mean body temperature for this group at the start of the experiment. Although this elevation was statistically significant at some time points ($p<0.05$, t-test) it was not considered to be of great physiological importance, as values for both control and 12% hypoxia animals remained within the normal limits for the guinea pig of 37.2 - 39.5°C (Harkness and Wagner, 1989).

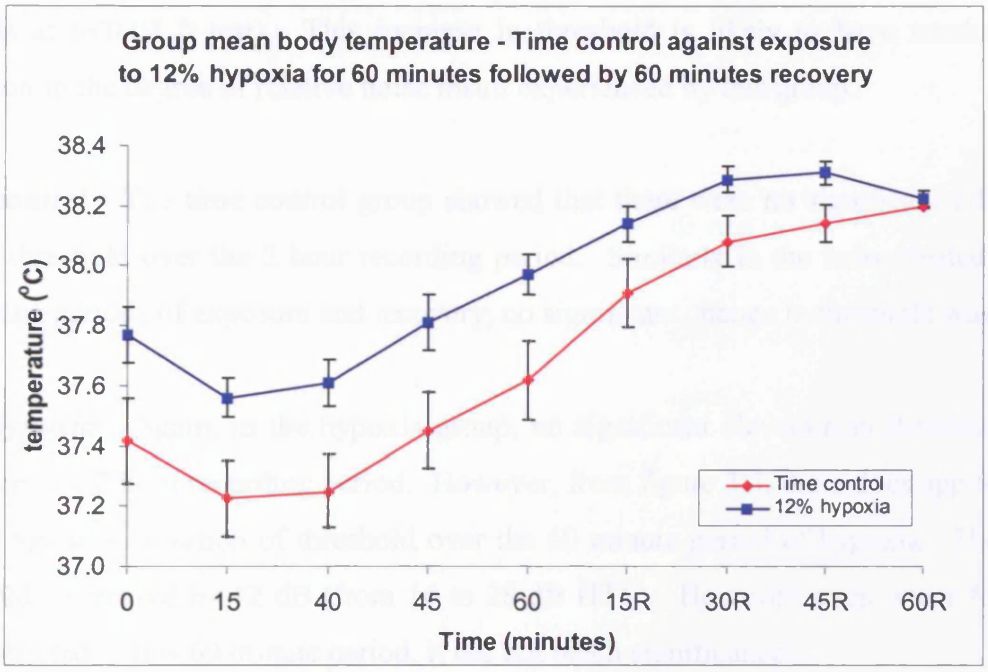


Figure 3.4. The effects of 12% hypoxia on rectal temperature after a two hour period. Note that the plots for both groups in parallel shows some evidence of 'homeostatic hunting' around a set point between 37 to 38°C (R - denotes during recovery period). Significance values are not highlighted here due to the absence of biologically significant threshold differences.

3.4.3.4. The effects of 12% hypoxia on respiratory rate

The effects of 12% hypoxia on respiratory rate were not systematically measured but typically fell between 70 to 90 breaths/minute whilst under neuroleptanalgesia. This was well within the normal range for the guinea pig of 42 to 104 breaths/minute (Harkness and Wagner, 1989). More importantly, with this regime there was no evidence of respiratory distress such as an irregular or forced breathing pattern.

In summary, the results gained here on ECG, pulse rate and body temperature support the contention that 12% hypoxia did not have any profound effect on general systemic

physiological condition. This supported the initial hypothesis that this moderate level of hypoxia would be suitable for use in combination with acute moderate noise exposure.

3.4.3.5. The effects of 12% hypoxia, noise or NH on BAER threshold

Thresholds at T_0 - The effects of the three different treatment groups on BAER threshold are shown in figure 3.5, with the statistical analyses summarised in Appendix 1. The BAER mean thresholds prior to exposure (ie. T_0) for the noise and hypoxia groups were 16 and 12 dB HTL respectively, which were not significantly different from the control group value of 15±2.2 dB HTL. However, the animals used in the NH group did have a mean threshold at T_0 of 24±2.2 dB HTL, which was significantly different from the controls at $p<0.03$ (t-test). This increase in threshold is likely to have resulted in a reduction in the degree of relative noise insult experienced by this group.

Time control - The time control group showed that there were no significant effects on BAER threshold over the 2 hour recording period. Similarly in the noise treated group, during the periods of exposure and recovery, no significant change in threshold was seen.

12% Hypoxia - Again, in the hypoxia group, no significant elevation in thresholds was seen over the 2 hour recording period. However, from figure 3.5, there does appear to be a trend towards elevation of threshold over the 60 minute period of hypoxia. The mean thresholds increased by 12 dB (from 16 to 28 dB HTL). However, even when ANOVA was restricted to this 60 minute period, it did not reach significance.

Noise or NH - Similarly, for the noise and NH groups there was also a trend in modest threshold elevation of 10 to 12 dB over the exposure period, with a return to normal during the recovery period. Again, over both the 60 minute and 2 hour time periods, changes in mean threshold did not reach significance with the exception of NH at 2 hours ($F_{8,42} = 10.73$, $P<0.0001$). This single change is unlikely to be of biological significance.

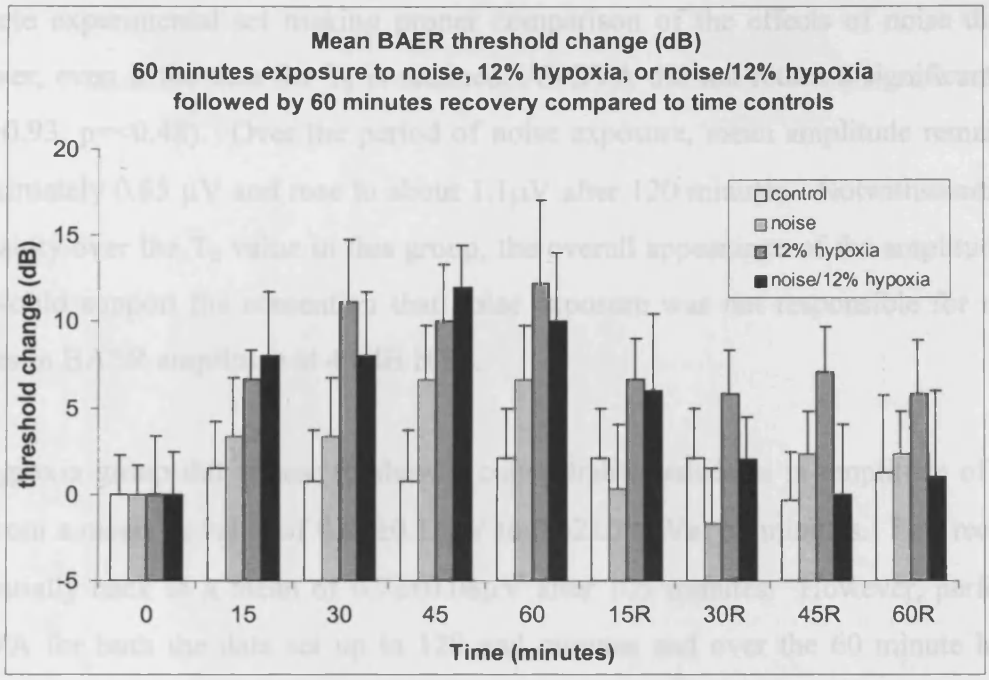


Figure 3.5. Mean BAER threshold shifts (±se) after exposure to noise, 12% hypoxia or noise/12% hypoxia seen over a 1 hour treatment period followed by 1 hour recovery. R - denotes recovery period.

3.4.3.6. The effects of 12% hypoxia, noise or NH on BAER wave I amplitude

The effects in each of the treatment groups on BAER amplitude over time are shown in Figures 3.6 and 3.7 obtained for 40 dB and 80 dB HTL respectively. Data was also obtained at other stimulus intensities between 10 and 80dB HTL, but the 40 and 80 dB stimulus levels were considered to adequately illustrate treatment effects over time. The responses at 40dB HTL were considered to reflect the activity of the metabolically more vulnerable active cochlear processes. Responses at 80 dB HTL would be more likely to reflect the predominantly passive BM driven cochlear transduction processes as described in section 1.3.1 (Puel *et al*, 1988 and Davis, 1983).

Amplitude at 40 dB HTL (within group analysis) - The mean T_0 value for the control group was 0.88±0.09μV. This decreased towards the end of the recording period to a mean value of 0.77±0.11μV at 105 minutes, but this reduction was not significant.

Unfortunately, the data gathered for the noise treated group at T_0 was suspected to be inaccurate (probably due to a transient error at this stimulus intensity setting over the period of experimentation with the noise treated animals only). This was reflected in the mean T_0 amplitude value of 0.5μV±0.1 compared with mean values of 0.76μV±0.16 to 1.1 μV±0.16 for the hypoxia and NH groups. This was only realised after conducting the

complete experimental set making proper comparison of the effects of noise difficult. However, even if the data for T_0 is retained, ANOVA did not return a significant value ($F_{8,46}$=0.93, p=<0.48). Over the period of noise exposure, mean amplitude remained at approximately 0.85 μV and rose to about 1.1μV after 120 minutes. Notwithstanding the uncertainty over the T_0 value in this group, the overall appearance of the amplitude over time would support the contention that noise exposure was not responsible for marked changes in BAER amplitude at 40 dB HTL.

The hypoxia group did appear to show a considerable reduction in amplitude of nearly 50% from a mean T_0 value of 0.81±0.15μV to 0.42±0.1μVat 60 minutes. This recovered substantially back to a mean of 0.72±0.08μV after 105 minutes. However, performing ANOVA for both the data set up to 120 and minutes and over the 60 minute hypoxia period did not reach significance.

In contrast, the effect of NH on amplitude at 40dB was highly significant. The T_0 mean of 0.86±0.17μV (n=5) dropped by nearly 70% to 0.27±0.045μV at 60 minutes. Notably, the greatest effect was attained after 15 minutes, also reaching significance at this time point. Recovery at 120 minutes was apparent with a mean amplitude value of 0.65±0.14μV. Post hoc comparison by Dunnett's test revealed that the mean values during exposure were all significantly reduced at $p<0.05$ (figure 3.6).

Across group analysis - ANOVA across groups revealed that there were no clear evidence of significant effects seen after 15 minutes. At 60 minutes the difference in means between groups was, however, highly significant ($F_{3,29} = 6.7$, $p<0.001$). Post hoc comparison of means by Tukey's test, revealed that the differences were significant between control and NH as expected. More specifically, the difference between noise and NH was also significant ($p<0.05$) indicating some degree of synergy. This was also evident in the relative percentage decreases at 15 and 60 minutes, which were greatest for the NH group (58 and 67% respectively).

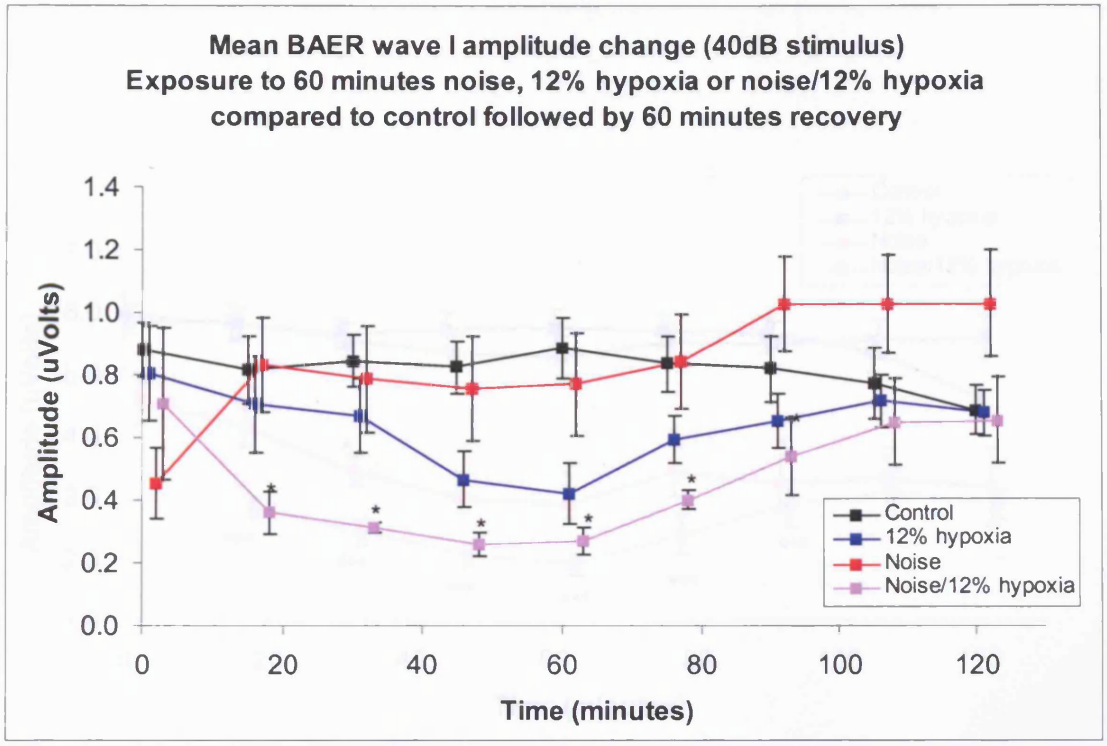


Figure 3.6. Mean BAER wave amplitude at 40dB HTL after exposure to noise, 12% hypoxia or noise/12% hypoxia seen over a 1 hour treatment period followed by 1 hour recovery (*=$p<0.05$) NB. The effect of leaving out T_0 for the noise exposed group still showed noise exposure not to have a significant effect. Note the greatest effect on the noise/hypoxia group was over 15 to 60 minutes. The difference between individual means in the noise and noise/hypoxia groups were also significant over 15 to 90 minutes at $p<0.05$. This was evidence of synergy between noise and hypoxia.

Amplitude at 80dB HTL (within group analysis) - The changes in mean amplitude in each treatment group are plotted in Figure 3.7. Again, no significant changes were seen in the time control group. Mean T_0 amplitude was 5.9±0.34μV compared to 5.35±0.31μV over the recording period. Compared to the T_0 mean value, no significant changes in amplitude over time were evident following 12% hypoxia exposure. The 12% hypoxia T_0 mean of 5.88±0.23μV was also comparable to the time control T_0 mean value.

The mean T_0 amplitude for the noise group was 4.61±0.42μV, and there were significant changes in this group over the recording period. Post hoc comparisons showed decreases to be significant ($p<0.05$ to 0.01) over 30 to 90 minutes. Mean amplitudes over this period ranged from 2.98±0.22 to 3.23±0.47 μV.

The effect of NH on amplitude was again highly significant. At T_0 the mean amplitude was 5.19±0.34 μV. Post hoc comparison with this value found reduction in amplitude was highly significant ($p<0.001$) for the whole of the recording period. Interestingly, this plot also shows that at 60 minutes the decreases in amplitude appear to be levelling off.

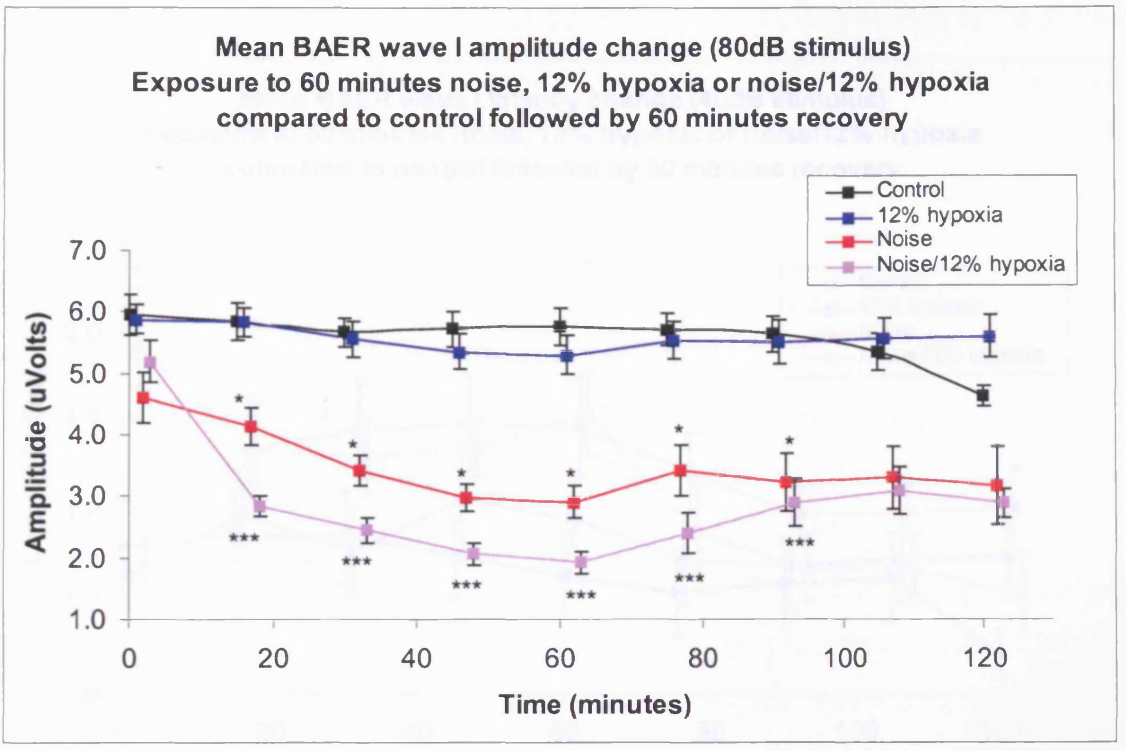


Figure 3.7. Mean BAER wave amplitude at 80dB HTL after exposure to noise, 12% hypoxia or noise/12% hypoxia seen over a 1 hour treatment period followed by 1 hour recovery. Over the whole recording period both noise and NH amplitudes were significantly reduced: Noise $F_{8,36}=2.45$, $p<0.05$ and NH $F_{8,42}=10.73$, $p<0.001$ (*=$p<0.05$, *** =$p<0.001$, t-test).

Across group analysis - The evidence for synergy between noise and 12% hypoxia is more clearly seen at 80 dB HTL, with the very marked percentage decreases amplitude reflecting the clear interaction between the two. At both 15 and 60 minutes, across group ANOVA yielded $P<0.0001$. Figure 3.7 very clearly shows the synergistic interaction of noise with hypoxia, which was further borne out by Tukey's post hoc comparisons of means with reduction of NH amplitude significantly greater ($p<0.05$) than noise alone. It is also noteworthy that a considerable majority of the synergistic effect of NH (ie. a drop of 45 %) was seen after 15 minutes. This represents about 70% of the total drop seen at 60 minutes.

3.4.3.7. The effects of 12% hypoxia, noise or NH on BAER wave I amplitude

As for BAER wave I amplitude, figures 3.8 and 3.9 summarise the results obtained for the effects of the four treatment groups on BAER wave I latency at 40 and 80 dB HTL.

Latency at 40 dB HTL (within group analysis) - The mean control group latency at T_0 was 1.7 ms ± 0.05 (n=13) and did not significantly change over the period. Similarly, the hypoxia group mean latency at T_0 was 1.73 ± 0.05 (n=10) and remained unaffected over recording.

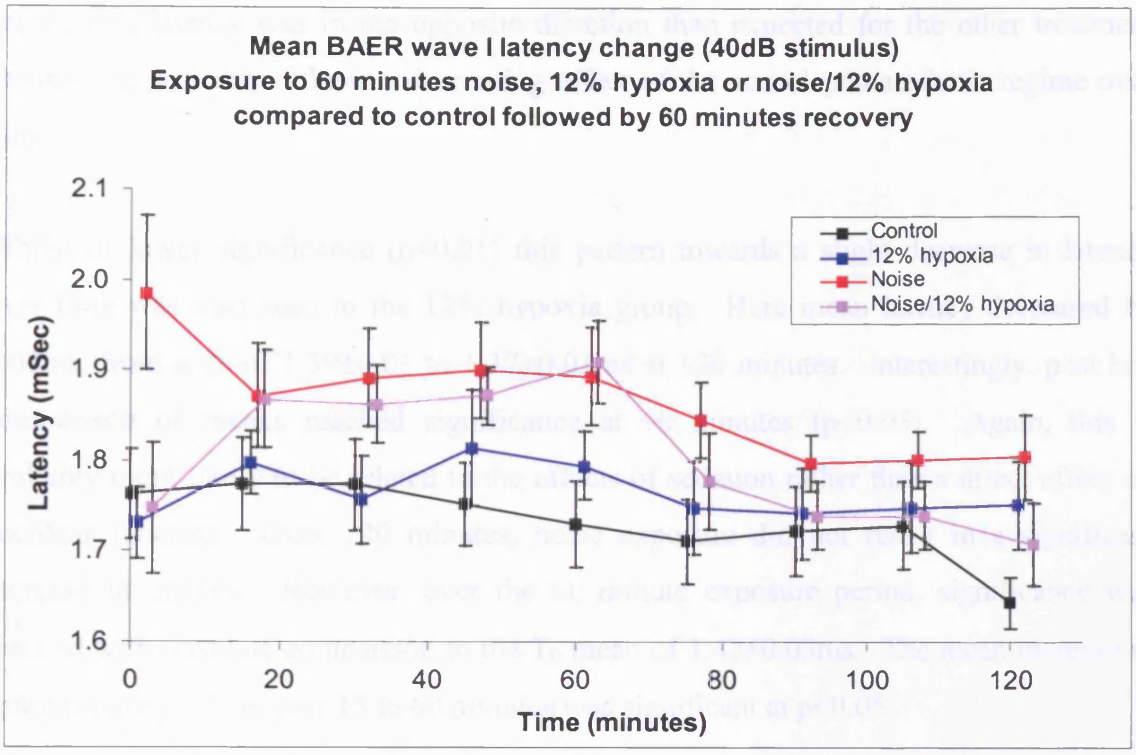


Figure 3.8. Mean BAER wave latency at 40dB HTL after exposure to noise, 12% hypoxia or noise/12% hypoxia seen over a 1 hour treatment period followed by 1 hour recovery.

As pointed out previously, the T_0 values obtained at 40 dB HTL for wave amplitude in the noise group were not considered reliable. This was also reflected in the greater than expected T_0 latency values. Again, even if T_0 was included in the analysis, the latency changes over time are not significant. If the T_0 group is removed from the analysis, the overall significance decreased further.

For the NH group, overall significance was attained with ANOVA ($F_{8,35} = 2.33$, $p<0.05$). Curiously, a post hoc t-test of the individual time points did not reach significance. However, the ANOVA result could be taken as evidence that NH at least over its period of presentation resulted in a marginal, but significant increase in latency.

Across group analysis - Comparison between group mean latencies at 15 and 30 minutes was not significant, although there was a trend towards increased latency over the time of exposure for the noise and noise/12% hypoxia groups.

Latency at 80 dB HTL (Within group analysis) - Interestingly, in the control group at 80 dB HTL, a highly significant effect ($p<0.001$) over time was seen in the control group. This amounted to a physiologically small decrease of approximately 0.03ms from a mean

at T_0 of 1.42±0.009ms to 1.38±0.007ms at 105 minutes. This very modest but significant decrease in latency was in the opposite direction than expected for the other treatment groups, but probably reflects a decreasing effect of the neuroleptanaesthetic regime over time.

Whilst of lower significance ($p<0.01$) this pattern towards a slight decrease in latency over time was also seen in the 12% hypoxia group. Here mean latency decreased by 0.02ms, from a T_0 of 1.39±0.01 to 1.37±0.01ms at 120 minutes. Interestingly, post hoc comparison of means reached significance at 90 minutes ($p<0.05$). Again, this is probably more likely to be related to the effects of sedation rather than a direct effect on cochlear function. Over 120 minutes, noise exposure did not result in a significant increase in latency. However, over the 60 minute exposure period, significance was reached with post hoc comparison to the T_0 mean of 1.42±0.03ms. The mean increase to approximately 1.5ms over 15 to 60 minutes was significant at $p<0.05$.

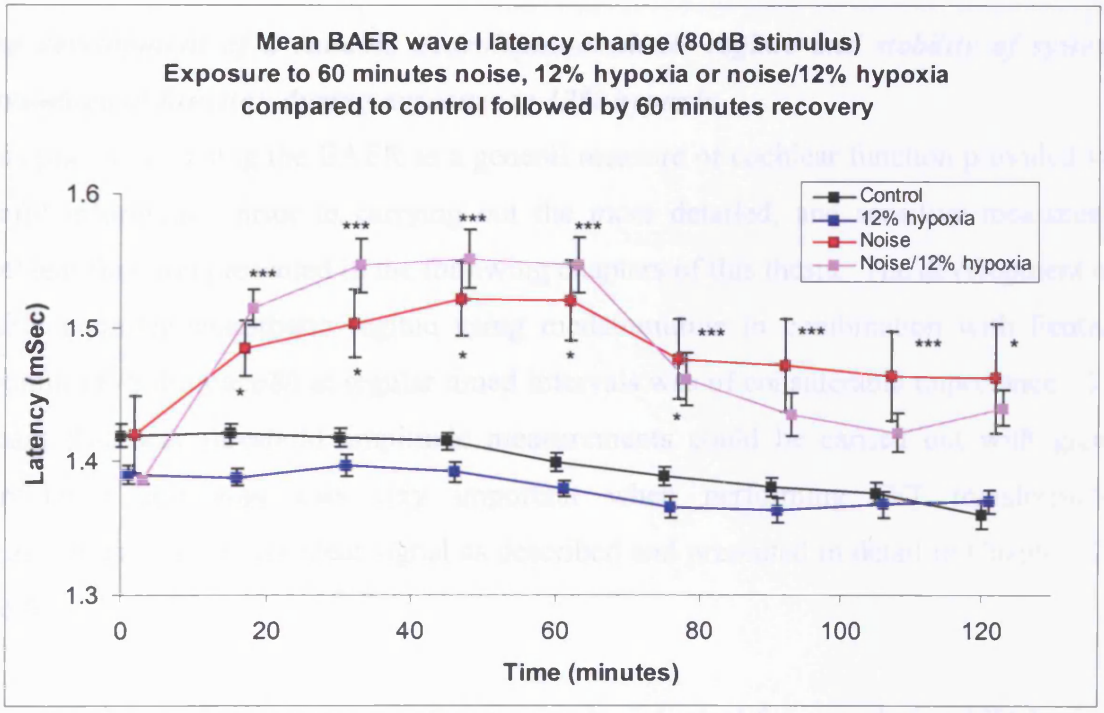


Figure 3.9. Mean BAER wave latency at 80dB HTL after exposure to noise, 12% hypoxia or noise/12% hypoxia seen over a 1 hour treatment period followed by 1 hour recovery. Only NH showed significant elevation over the 120 minute period ($F_{8,43}=13.61$, $p<0.0001$). The physiologically small but highly significant drift towards reduced latency for both the control and 12% hypoxia groups is not marked for significance (*=$p<0.05$, *** =$p<0.001$).

In contrast, NH treatment had a marked and significant effect ($p<0.001$) in causing an increase in wave latency. The T_0 mean of 1.39±0.003ms maximally increased to

1.55±0.02ms at 45 to 60 minutes. Over the treatment period, post hoc comparison of individual means were all highly significant ($p<0.001$). Partial recovery of mean latency was seen after 120 minutes but was still significantly elevated with a mean value of 1.44±0.014ms ($p<0.05$). As with BAER wave amplitude at 80dB HTL, the proportionately greatest increase in latency (ie about 80%) was seen 15 minutes into the period of exposure. After this, the increase appeared to rapidly approach an asymptote.

Across group analysis - The between group ANOVA at 15 and 60 minutes treatment were both highly significant ($F_{3,31}=33$, $p<0.0001$). Interestingly, the post hoc comparison at 60 minutes between control and noise means was significant at $p<0.05$. Compared to control, the mean NH values were significant at $p<0.0001$. The mean latency increases for the NH group were greater over the 60 minute exposure period than for the noise alone group but post hoc testing showed that this did not reach significance.

3.5. Discussion and conclusions

The development of a suitable neuroleptanaesthetic regime and stability of systemic physiological function during exposure to 12% hypoxia

This pilot study using the BAER as a general measure of cochlear function provided very useful information prior to carrying out the more detailed, and sensitive measures of cochlear function presented in the following chapters of this thesis. The development of a stable neuroleptanaesthetic regime using medetomidine in combination with Fentanyl (Domitor®/Sublimaze®) at regular timed intervals was of considerable importance. This meant that near threshold amplitude measurements could be carried out with greater confidence and was also very important when performing FFT transformation measurements of the cochlear signal as described and presented in detail in Chapters 2, 4 and 5.

The attention paid to monitoring of systemic physiological function during 12% hypoxia also afforded further confidence in utilising this as a potential synergising agent with noise. Whilst there was evidence of modest changes in systemic physiological function these occurred well within the normal ranges cited for the guinea pig. It was of interest to note that 12% hypoxia lead to a slight but consistent elevation in core temperature as shown in Figure 3.4.

Within group effects of noise, hypoxia, and NH in combination on the BAER

The results from this pilot study demonstrated that the control values for threshold and amplitude remained very stable over the recording period. Control latency showed a physiologically slight, but highly significant decrease over the recording period that was probably reflective of a decreasing effect of neuroleptanaesthesia over time.

One of the key results from this pilot study was that 12% hypoxia did not appear to significantly affect gross cochlear output as measured by BAER. This is of considerable physiological interest, as it suggests that at least over one hour, about 85 % haemoglobin saturation is adequate to supply the metabolic needs of the cochlea 'at rest'. This level is also adequate to support the short term driven activity of the cochlea as evidenced by the stability in amplitude measurement. As latency is a measure of CN function the absence of effect on the latency also suggests that the CN is unaffected by this level of oxygenation. Noise exposure significantly affected BAER amplitude and latency at 80 dB HTL but did not affect BAER threshold or output at 40 dB HTL. There was about a maximal 40% reduction in amplitude over the period of noise exposure, whilst latency increased by about 8% above the control value of 1.39ms.

This absence of an effect on the lower intensity part of the BAER most likely reflects the partial overlap between different spectra of the bandpass noise stimulus and the BAER click stimulus as shown in figure 3.10. The click stimulus elicited responses between 1-10 kHz with a peak about 4 kHz. In contrast, the noise stimulus was bandpass filtered between about 5-20 kHz. This would have led to a reduced effect of the noise on the cochlea at frequencies below 5 kHz. With higher 'click' stimulus levels, the effect of the noise on N_1 generation would be more marked, as more frequencies above 4 kHz would be recruited to produce an N_1 response at higher levels (Puel *et al*, 1988).

The effects of NH on the BAER were evident at both 40 and 80 dB HTL though again not at threshold and this was also likely to be due to the limited spectral overlap between click stimulus and noise mentioned above and shown in figure 3.10. Interestingly, for both noise and NH, the effect on both amplitude and latency seemed to reach maximal effect rapidly ie within the first 15 minutes or so and then approach a limit by about 60 minutes. This points to a steady state within the cochlea being reached in terms of

dealing with the increased metabolic demands due to the need to deal with the higher levels of glutamate recycling.

Across groups effects of noise, hypoxia, and NH in combination on the BAER

Across group analysis provided supporting evidence for a synergistic effect between noise and hypoxia particularly with regards the effect on amplitude. Significant differences between mean noise and NH values showed that decreases in amplitude were greater with NH than noise alone, whilst hypoxia alone had no effect. Interestingly, this synergistic effect did not appear to apply as strongly to BAER latency. At 40dB HTL, there was no evidence of any treatment effect but at 80 dB HTL it amounted to an increase between 0.1 and 0.15ms. There was a trend to a slightly increased latency with NH above noise alone, but this did not reach significance.

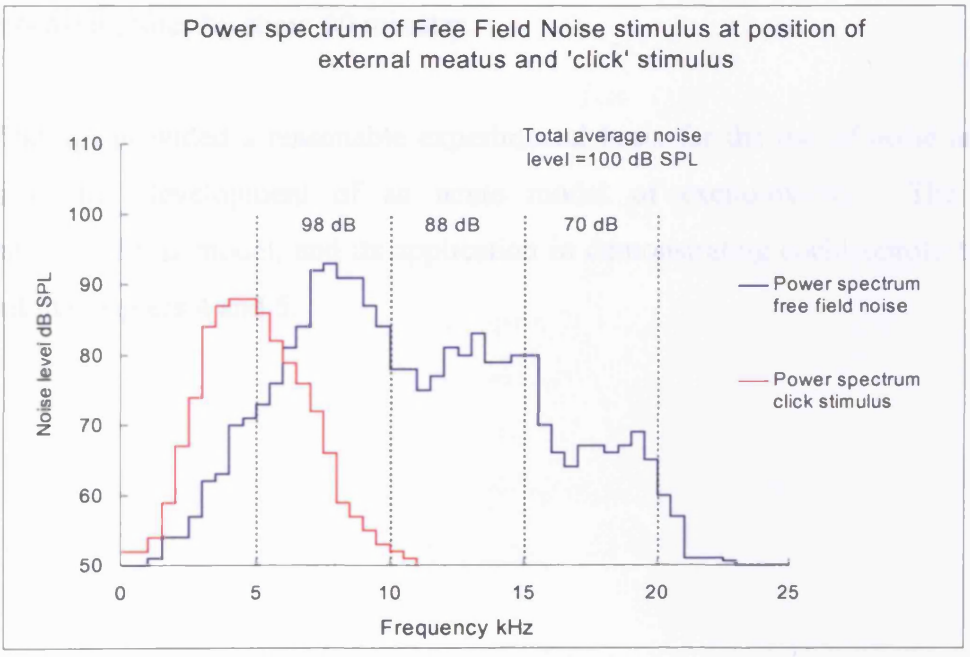


Figure 3.10. The power spectrum of both free field noise and the BAER 'click' stimulus at the position of the external meatus. Note the distinct difference in frequency spectra between the two stimuli.

In summary, the conclusions from this preliminary study were as follows:

- 12% hypoxia for one hour does not result in major changes in normal systemic function or the BAER.

- Noise/12% hypoxia (NH), in combination, result in a synergistic effect on the suprathreshold amplitude response.

- Both noise and NH cause a moderate but highly significant increase in latency, though there does not appear to be a very marked synergistic interaction between noise and hypoxia on latency.

- The majority of effect occurs within the first 15 minutes of exposure, after which the effects asymptotes by about 60 minutes.

These findings provided a reasonable experimental basis for the use of noise and 12% hypoxia in the development of an acute model of excitotoxicity. The further developments of this model, and its application in demonstrating cochleoprotection, are presented in Chapters 4 and 5.

Chapter 4. The effects of noise, 12% hypoxia or noise/12% hypoxia (NH) on compound action potential (CAP) and ensemble spontaneous CN activity (ESAC)

4.1. General Introduction

The purpose of the work presented in this chapter was to attempt to establish an acute model of cochlear excitotoxicity. If this was successful, it was intended to use the model in demonstrating pharmacological protection against any excitotoxic component in noise induced hearing loss (NIHL).

The primary focus was to investigate gross electrophysiological changes that occur after acute bandpass noise exposure, leading to the proposed excitotoxic events previously described by Puel *et al* (1991), Liu and Fechter (1995), Chen *et al* (2001) and Fechter *et al* (1988, 2000). From this, the model would also be used to obtain much more specific tonotopic information about the extent and nature of the noise and NH trauma, especially at the level of the CN. Although requiring surgical exposure, direct gross electrode recordings from the cochlea afford considerably greater sensitivity and specificity than BAER recordings. Compound action potentials (CAP) N_1 thresholds and input/output functions for amplitude and latency were used as the principle electrophysiological endpoints. Four frequencies were investigated 8, 16, 24 and 30 kHz, with the upper frequency range deliverable being software-limited.

The experimental design in this chapter was based on the results from the exploratory BAER experiments in Chapter 3. These dealt with changes in the cochlear evoked responses to simple 'click' stimuli before during and after 60 minutes exposure to 12% hypoxia, noise and noise/12% hypoxia (NH). The BAER results showed that the near maximal changes in gross cochlear function occurred within the first 15 minutes of exposure to noise or NH. Consequently, this period of insult was adopted for the model for the experiments in this chapter. Whilst the upper frequency limit of the guinea pig is about 64 kHz (Heffner *et al*, 1971), the 30 kHz tonotopic location in the guinea pig is sufficiently close to the basal end as to provide information about frequency specific vulnerability (Robertson, 1984). Given the greater vulnerability of high frequencies to ototoxins (Monsell *et al*, 1997) there has been curiously little investigation into the effects of noise on these higher frequencies in animal models. Consequently, in this study it was

considered to be a particularly important question to address further, especially with noise and hypoxia in combination.

To further characterise any excitotoxic effects, the relatively novel technique of recording ensemble spontaneous CN activity (ESAC) was also employed, to provide a gross measure of changes in CN activity before, during and after exposure to insult. As discussed in Chapter 1, the higher frequency component of the ESAC power spectrum reflects afferent CN activity. The study of ESAC would hopefully provide a measure of how CN activity was modulated by excitotoxicity (Cazals *et al*, 1998 and Cazals, 2000). The quantitative power measurements would also provide a way of assessing metabolic demand of the CN during periods of both silence and auditory insult.

4.2. Background to literature on experimental models of NIHL

There is a very large literature base on the effects of noise on hearing, which has been an active field of study since the 1950's to the present. One of the curious features in the experimental literature is that there appears to be no systematic approach to investigation. In particular, there do not appear to have been many coherent, species specific investigation of dose-effect relationships (Sullivan and Connolly, 1988). Moreover, comparison between studies is often problematic due to the use of different species, the main ones being chinchilla, rat and guinea pig. Probably the most important studies carried out were detailed single CN fibre studies, but these were carried out in the cat, and only using low to mid-frequency narrow band moderate noise (Kiang *et al*, 1976, Liberman, 1984, Liberman *et al*, 1984a-b and Liberman & Dodds, 1987).

Perhaps, understandably, most of the work has been driven by investigation of those levels, durations and acoustic frequencies encountered regarding development of NIHL in man. This has tended to mean the use of noise stimuli typically encountered in either military or industrial environments. This has involved use of low to mid frequency stimuli (0.5 – 4 kHz) with levels between 90-120 dB SPL delivered over hours or days (Salvi *et al* 1979 & 1983, Morest 1982, Liberman *et al* 1982 & 1984, Sullivan and Connolly 1988, Clark 1991, Boettcher *et al* 1992). Other studies have utilised very loud (150 to 160 dB SPL) impulse stimuli, presented over a few minutes, to mimic the effects of gunfire (Henderson *et al*, 1974 and 1977, Hamernick and Henderson, 1976, Hamernick *et al*, 1980, Henderson and Hamernick, 1986).

The brief review here focuses on the main findings from these previous studies, with most relevance to interpreting the role of noise and NH in generating damage at the sites of principle interest to this study, ie. the IHC and CN.

4.3. Noise stimulus in animal models of NIHL

Noise-induced hearing loss (NIHL) is one of the most common occupational health hazards in industrial societies (Boettcher *et al*, 1998) and it continues to remain the most common occupational disease in the United States (NIOSH, 1996).

4.3.1. Temporary and permanent threshold shift

It has been known for years that excessive exposure to noise results in both temporary and permanent threshold shifts (TTS and PTS respectively). Excessive exposure to noise results in either direct mechanical damage to the sensitive cochlear structures or from metabolic overload due to overstimulation (Prasher, 1999). The process of NIHL involves not only the sensory cells but also the supporting cells, nerve fibres and in more extreme cases, the vascular supply. Consequently, the loss due to either TTS or PTS can involve mixed sites in the cochlea.

Clinically, the best known evidence of NIHL is an increase in auditory thresholds. This may manifest itself as either a temporary threshold shift (TTS) where it recovers within hours or days, or a permanent threshold shift (PTS) where unrecoverable damage of the cochlea occurs (Quaranta *et al*, 1998). In the experimental model developed here, the loss aimed for was intended to mimic moderate to severe TTS.

4.3.2. General characteristics of noise in experimental studies

In information theory, noise is considered simply as any unwanted signal (Barlow and Mollon, 1982). This definition can also apply to acoustic signals so that in effect, a single pure tone if defined by the experimenter as 'unwanted', would constitute noise. More generally, in auditory studies noise refers to an acoustic signal of mixed frequency components typically falling within a defined frequency range. These may have varying intensity, but always have random phase (Bruel and Kjaer, 1998).

Perhaps the best known kind of acoustic noise is white noise. This is called white noise as a parallel to light signals. Theoretically, averaged over time, all frequencies are present at *equal intensity,* but are in random phase (Meade and Dillon. 1991). White noise is not usually favoured in noise experiments. Most studies have used *band pass* filtered white

noise in an attempt to focus damage over one part of the cochlea (Kiang *et al*, 1976, Salvi *et al*, 1979, Puel *et al*, 1988, Sullivan and Connolly, 1988, Attias *et al*, 1990, Fechter *et al*, 2000, Chen *et al*, 2001). The *band pass* refers to the spectrum limits over which the great majority of the energy is delivered. For example a band pass of 5-10 kHz would mean that the noise would fall between 5-10 kHz with little signal falling outside this range.

In Chapters 4 and 5, white noise was filtered between about 5 and 20 kHz prior to delivery to the free field speakers. Given the properties of the speaker system and the acoustic properties of the hypoxia chamber, the appearance of the signal at the external meatus was as shown in figure 4.1. This shows that the band pass signal could be divided further into three principle spectral bands at 5-10 kHz, 10-15 kHz and 15-20 kHz, with the first band showing maximal power at about 98 dB SPL

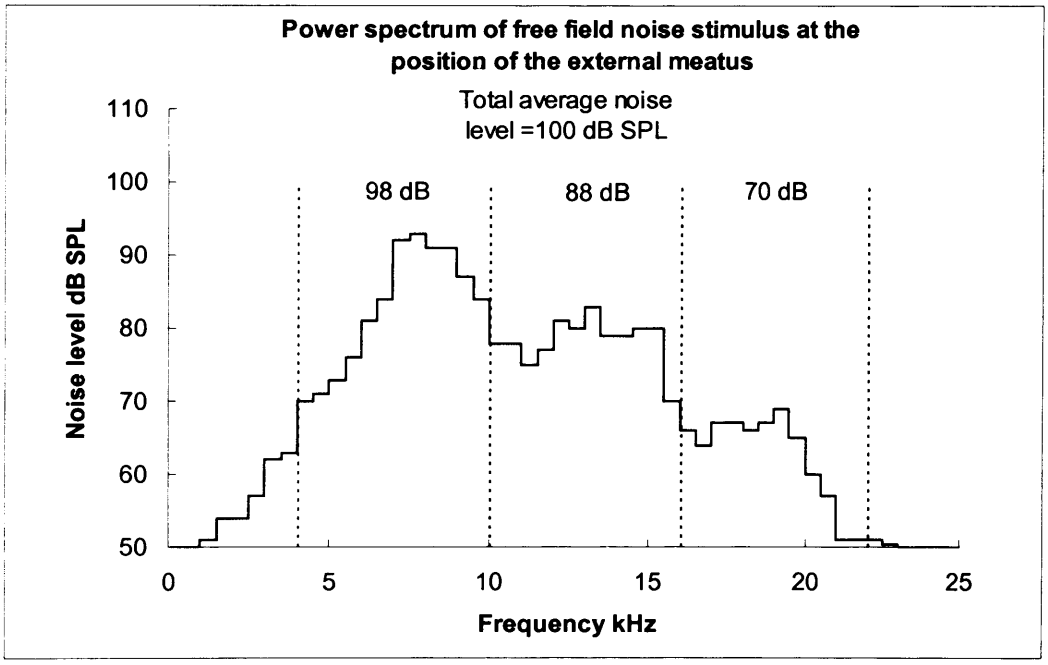


Figure 4.1. Power spectrum of free field noise generated in this study, in response to band pass filtered noise (100dB SPL) at the level of the external meatus. Note that because of the log-log scale, the majority of power is contained in the 5-10 kHz band. The power over the 10-20 kHz region on contributes a small proportion of the energy delivered to the cochlea.

Whilst use of this stimuli had some limitations, it did act to provide differential noise insult to the cochlea so that a gradient of damage could be generated in the direction of apex to base. As no one part of the stimuli was much above 90 dB SPL (figure 4.1) this also lowered the probable contribution of physical disruption. The 'smearing of insult' also meant a maximal number of fibres would be firing at or near their maximal discharge

rate, and thus increasing the likelihood of excitotoxic damage (Liberman and Dodds, 1984a-b).

4.3.3. Noise power calculations using a log-log scale

It is important to note that both the dB and frequency scales in figure 4.1 are Log-Log axes. This means that the dB power calculations from the plot can appear somewhat counter-intuitive. The Log–Log nature of the calculation can be illustrated by considering a single frequency tone at 1 kHz with an SPL of 100 dB. If we add another frequency, say 1.001 kHz at 100dB SPL, then the overall dB SPL is 103 dB SPL. This is because +3 dB SPL represents a *doubling* of power. If the power of these two waves is used as the initial reference intensity, then four waves at 100 dB SPL would give 106 dB SPL (again +3 dB being a *doubling* in power). So, for equal intensity waveforms of any frequency, the overall power is given by the equation;

$$Averaged\ overall\ Power = 10\ log_{10}(2^N) + X\ dB\ SPL$$

where X is the reference dB SPL pressure for a single wave, and N represents the number of wave forms present in the signal at X dB SPL. The Log-Log scale also means that the relative contribution of adding a signal at say 80dB SPL to a signal of 100 dB SPL is negligible. Proportionately, the 80dB SPL signal would contribute 1% of the total power and would come to about 100.05 dB SPL (Bruel and Kjaer, 1998).

4.4. NIHL - Relationship between intensity and duration

Noise can be characterised according to figure 4.2 below which shows the relationship between the magnitudes of sound pressure, power SPL and perception of loudness. From this is can be seen that in man, constant exposure to levels below about 80 dB SPL over about 0.5 to 8 kHz, are not considered to pose any real risk of permanent hearing loss (Mills and Going, 1982). Above this level, there is proposed to be a log/log relationship between intensity/duration and the risk of both TTS and PTS (Miller, 1974) as shown in figure 4.3. As a consequence of this, many studies have utilised moderate to very high levels of noise and exposure periods have been over hours or spread over days in order to generate a profound model of NIHL.

These kinds of exposure are very likely to consist of mixed site PTS, and provide little or no opportunity for identifying the earlier stages of development of excitotoxic damage.

However, as can be seen from figure 4.3, quite substantial threshold elevations of about 20 dB can be achieved with 100 dB SPL stimuli after 15 minutes. As supported by the preliminary results in Chapter 3, this degree of elevation is likely to be due to moderate acute excitotoxic damage.

Sound pressure ($N\ m^{-2}$ or Pa)	Power (intensity) ($W\ m^{-2}$)	Sound pressure level (dB SPL, i.e. referred to 20 μPa)	Examples and some effects (approximate only)
200	100	140	Jet engine; over-amplified rock group; threshold for pain
20	1	120	Damage to cochlear hair cells
6.32	10^{-1}	110	Threshold for discomfort
2	10^{-2}	100	Motor cycle engine
			Orchestra
6.32×10^{-1}	10^{-3}	90	*fff*
2×10^{-1}	10^{-4}	80	*ff*; busy traffic; shouting
2×10^{-2}	10^{-6}	60	*mf*; normal conversation
2×10^{-3}	10^{-8}	40	*pp*; quiet office
6.32×10^{-4}	10^{-9}	30	*ppp*; soft whisper
2×10^{-4}	10^{-10}	20	Country area at night
2×10^{-5}	10^{-12}	0	Threshold of hearing of young person at 1–5 kHz
6.32×10^{-6}	10^{-13}	−10	Threshold of cat's hearing (1–10 kHz)

Figure 4.2. Relationships between the magnitudes of sound pressure, power (intensity) and SPL. Taken from Barlow and Mollon, 1982.

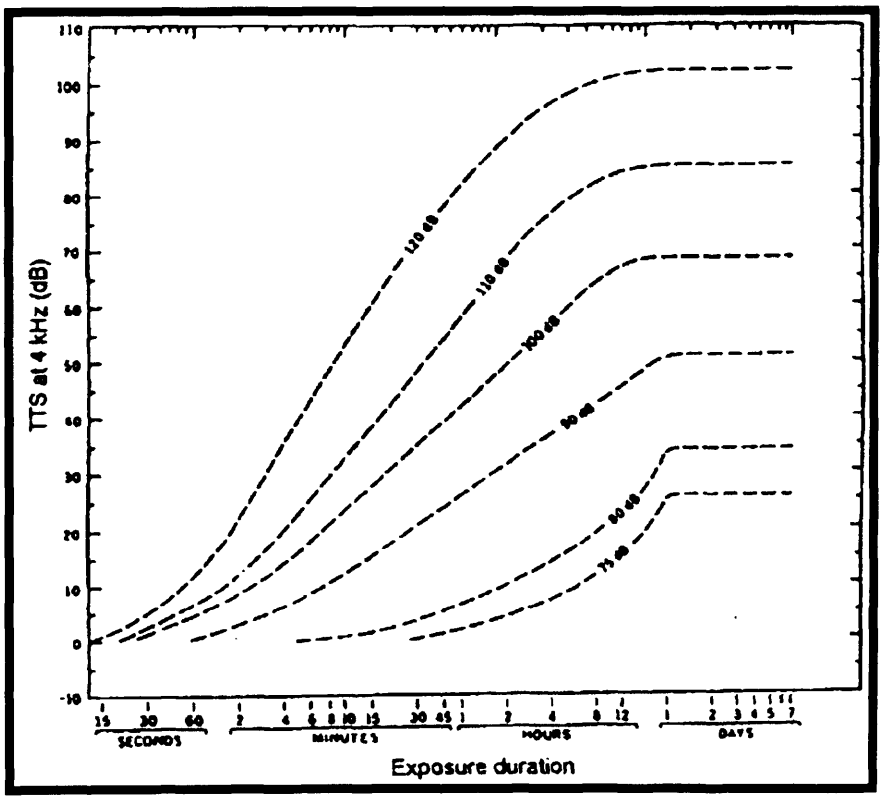


Figure 4.3. Hypothetical TTS at 4 kHz as a function of exposure duration and intensity for noise of different intensities. Adapated from Miller, 1974.

4.5. The sites and mechanisms involved in NIHL

The current thinking on sites and mechanisms of damage leads to three main modes of action being involved in the generation of NIHL. These comprise; direct physical damage to the structures in the scala media, disturbance to biochemical processes in the organ of Corti, leading in particular to generation of free radicals, and sequelae of damage due to excitotoxicity at the afferent synapse/CN caused by excess release of glutamate by the IHC. These are considered individually below, with particular consideration given to changes in electrophysiological measures of cochlear and eighth nerve function.

4.5.1. Physical damage

As the levels and duration of noise used in this study were chosen to minimise their potential contribution to physical damage, coverage of the literature here has been limited. More detailed consideration of physical damage contributing to NIHL especially at higher noise levels, is described elsewhere (Mills *et al*, 1979, Liberman *et al*, 1982, Henderson & Hamernick, 1995).

Dimensions of noise induced physical disruption in the cochlea - The functional microstructure of the cochlea is optimally designed to transduce very low sound energies between 10^{-17} W to 10^{-8} W ie –10 dB to 80dB SPL (Moore, 1995). Above 10^{-6} W and up to 10^{-4} W, these very large densities of acoustic energy affect the very precise alignment of microstructures. An energy density of 10^{-6} W may not seem very great (ie 1 microwatt) but it should be borne in mind that this energy is acting on structures with dimensions ranging from a few nanometres (ion channels) up to tens of microns in the Organ of Corti (Ades and Engström, 1974, Pickles, 1991). At intensities between 100-120 dB SPL the dimension of the BM travelling wave itself has been measured at 100-1000 nm or 0.1–1μm in the normal cochlea (Johnstone *et al.* 1986). It can be imagined that over time this level of displacement would lead to damage to the stereocilia.

Vulnerability to noise damage of separate sites within the cochlea - In brief, the order of susceptibility to physical damage is as follows; OHCs and their supporting cells, IHCs and their supporting cells, afferent fibres and finally the stria vascularis in extreme cases. Electron microscopy studies have revealed that the stereocilia of both OHCs and IHCs are vulnerable to acute noise levels above 100-110 dB SPL (Henderson and Hamernick, 1995). There also appears to be a clear pattern in which the hair cells lose functional integrity following severe prolonged noise exposure (Morest, 1982, Sullivan and

Connolly, 1988). OHCs are usually affected first with the order of row 1→row 2 row→3 followed by the IHCs. Following IHC death, loss of the afferent nerve fibres is also seen.

Following a traumatic noise exposure, damage or loss to OHCs is invariably greater than for IHCs. The OHC stereocilia seem to be particularly vulnerable to disarray. After acoustic trauma the stereocilia lose their rigid straight appearance and become 'floppy' and detached from the tectorial membrane. The OHCs also experience a tremendous shearing force at the level of the stereocilia whereas IHC stereocilia are stimulated by viscous drag though at higher noise intensities would also come into contact with the tectorial membrane. In addition, OHCs are far more exposed to noise due to their structural location compared to IHCs which are protected on all sides by supporting cells as shown in figure 1.3 (Henderson & Hamernick, 1995). Lastly, the OHCs are closer to the point of maximal BM travelling wave displacement than are IHCs (Henderson & Hamernick, 1995).

With intense noise exposure, physical damage is likely to occur during the exposure period (Puel *et al.*, 1988, 1998). However, subsequent physical damage can also take place over the longer term. This is due to a gradual loss of structural integrity of the Organ of Corti following apoptotic cell death and subsequent scarring (Henderson & Hamernick, 1995). Damage can also occur to the IHCs with a concomitant loss of inner pillar cells. There is also degeneration of afferent auditory nerve fibres reflected in losses of spiral ganglion cells and morphologic changes (Morest, 1982).

In physiological terms, physical disruption results in damage to the active amplification and attenuation processes, which is reflected in proportionate threshold elevations and deficit in performance of the cochlear amplifier (Kiang *et al*, 1986). Again, in this model developed by Kiang *et al* (1986) the levels of severe physical disruption described above are unlikely to occur although some stereociliary damage is possible (Henderson and Hamernick, 1995). Maximal displacement of the BM would not be expected to exceed 100 nm (Johnstone *et al.* 1986).

4.5.2. Biochemical damage

Recently, the biochemical changes in the cochlea due to noise damage have been investigated, and are reviewed by Henderson *et al* (1999) and Altschuler *et al* (1999). These findings are of considerable interest and have shown the cochlea responds in similar ways to other cells when dealing with stress. However, direct relevance to the acute model developed in this thesis is unclear, as *maximal* biochemical damage seems to be most prevalent about 12 to 48 hours after the noise insult (Henderson and Hamernick, 1995). As measurements in this thesis were made directly after acoustic trauma, the contribution of biochemical damage to observed functional deficit is not clear.

One of the key findings from this work has been identifying the generation of free radicals in the cochlea following acute high levels of noise exposure (Hu *et al*, 1997, Ohinata *et al*, 2000). It was shown that 105 dB SPL over 4 hours caused an increase of free radical species in the cochlea (Hu *et al*, 1997) but the biochemical damage due to these free radicals could be ameliorated by introduction of a scavenger (R-phenylisopropyladenosine; R-PIA). An interesting observation was that R-PIA seemed to be most active about 3 to 4 days after the noise insult indicating a delayed response to biochemical insult. In addition, the cellular antioxidant glutathione administered prophylactically has been shown to limit NIHL (Ohinata *et al*, 2000) but this study did not provide any indication as to the onset of biochemical damage.

More generally, the biochemical changes in the cochlea appear to follow those arising from a decline in aerobic energy metabolism and a possible cascade of metabolic and ionic changes. This in turn leads to loss of Ca^{2+} homeostasis and eventually, irreversible injury (Billet, 1989). Noise exposure increases demand on the cochlea to such an extent that, especially in combination with hypoxia, damage would more than likely be greatly accentuated. These Ca^{2+} dependent pathways are summarised in figure 4.4 include activation of nitric oxide syntheses (NOS), lipases, especially phospholipase A_2, protein kinases, endonucleases and proteases (Dugan *et al,* 1994).

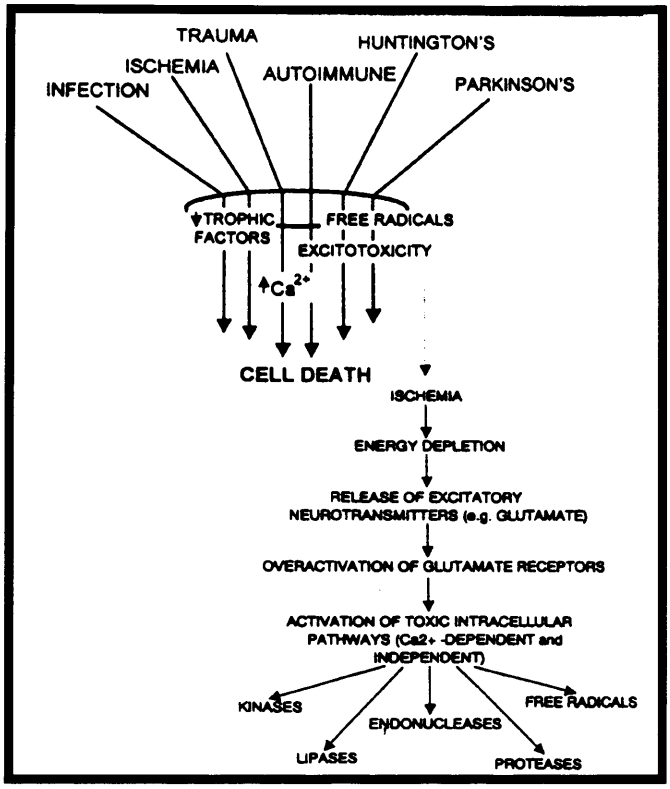


Figure 4.4. Various types of nervous system injury. The proposed events for an ischaemic episode have been shown here (taken from Dugan and Choi, 1994).

Noise exposure does have immediate effects on cochlear metabolic demand and these are more relevant to interpretation of the experiments in this thesis. Ryan (1988) showed that moderate noise activity caused substantial increases in metabolic demand in the cochlea (figure 4.5). The stria vascularis, responsible for generating the EP, showed an increase of approximately 30%. In contrast, the increase in metabolic demand in the IHCs was approximately 160%. The greatest increase, however, was seen in the CN (not shown) which went up by 220%. These findings highlight the metabolic vulnerability of the IHCs and CN to noise insult and reinforces the idea that noise in combination with moderate hypoxia will act preferentially at these sites.

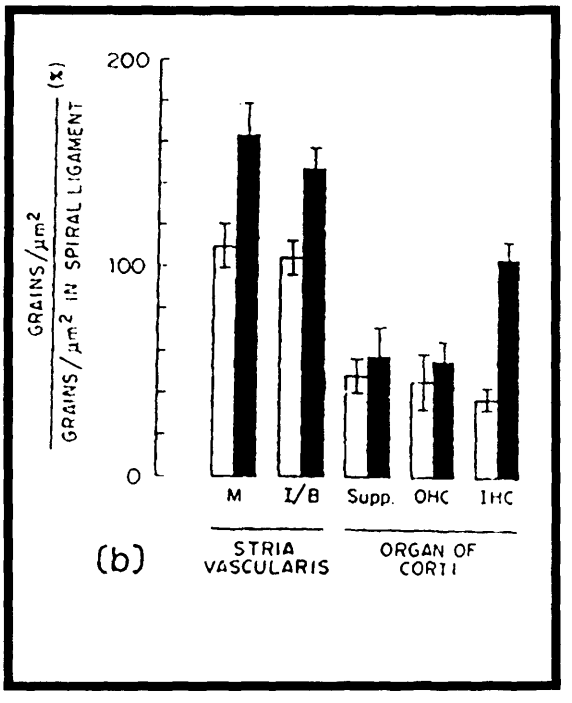


Figure 4.5. The relative metabolic activity of cochlear structures in silence (open bars) and during noise stimulation (85 dB SPL white noise). Increase in activity was measured by 2-deoxyglucose uptake in the different cell types. Supp. = supporting cells. Adapted from Ryan, 1988.

4.5.3. Excitotoxic damage

Excitotoxic damage within the cochlea has been reviewed in detail in Chapter 1. However, a brief consideration is given here on the additive effects of excitotoxicity to physical and biochemical damage, causing performance deficit in the cochlea. Importantly, excitotoxic damage could result in either TTS or PTS depending on the severity of damage to the afferent fibres (Puel *et al* 1998, 1999). As a consequence, Puel *et al* (1999) proposed that a TTS or PTS component, contributed by CN damage, was due to the degree of NMDA involvement in the carriage of Ca^{2+} current. With TTS, they also proposed that damage was due to excessive currents only being carried by AMPA receptors (Puel *et al* 2002). The resultant outcome would be that TTS diminished when the CN fibres recovered, and when fibres were lost they would contribute to PTS. This loss would not have a direct effect on OHC function *per se,* but resulted in hearing deficit due to loss of an intact signal line. This model was, however, developed in part by direct pharmacological insult, using AMPA to saturate CN glutamate receptors. Selvadurai *et al* (2000) and Chen *et al* (1999, 2002) have shown that the NMDA antagonist MK-801, protected against noise/hypoxia induced hearing loss, having a significant protectant effect against both TTS and PTS.

This shows that the important question of the relative contribution made by the glutamate receptor types to excitotoxicity in the CN is still not fully characterised. This is of central relevance in the future development of the most appropriate therapeutics.

4.6. Physiological changes in the cochlea following noise exposure

There have been a substantial number of studies investigating the effects of moderate (90 dB SPL) to intense noise (140+dB SPL) on cochlear physiology. Again, there appear to have been relatively few systematic investigations looking at the sites of damage in the cochlea. Only one of the papers reviewed focused on differentiating between putative cochlear sites of action of acute moderate noise (Puel *et al.* 1988).

Effects of noise on CN fibres, and cochlear anatomy studies by Liberman et al - Probably the most important detailed study at the single unit level in the cat was reported in a series of landmark papers by Liberman (1984), Liberman *et al* (1984a-c) and Kiang *et al* (1986). In this series of experiments the authors correlated severity and pattern of performance deficit in the nerve with patterns of both OHC and IHC damage. This enabled them to make inferences about the contributions to the deficit by the different sites. In these experiments, each animal was exposed to single narrow band noise stimuli (50 Hz intervals below 3 kHz and 500 Hz intervals above 3 kHz) delivered at levels of 110-120 dB SPL for two hours. The noise centre frequency was systematically varied in each animal so that all the data went to constructing a tonotopic damage map. After at least one month to allow the progression of lesioning, single unit recordings were then made. Effectively, four classes of damage were identified, as judged by changes seen in the single fibre FTC (see figure 1.10) and SR:

Class 1 - Threshold elevation of the FTC tip (about 20-40 dB SPL) with some loss of sharp tuning and mild to marked tail hypersensitivity, correlated with modest (typically 1st row) OHC stereociliary damage.

Class 2 - A poorly tuned high threshold (40-60 dB SPL) tuning curve with no tip and a hypersensitive tail correlated best with severe OHC damage or loss, but no IHC loss.

Class 3 - Severe damage to both OHCs and IHCs correlated with a very high threshold (>70-80 dB SPL) and very poorly tuned FTC.

Class 4 - Elevation of both the FTC tip and tail threshold, but with no loss of tip selectivity principally correlated with IHC stereociliary damage.

Effects on driven and spontaneous CN activity - There were varying degrees of depression in the transfer function related to OHC damage. In the more severely damaged areas there was an initial depression which then showed a more rapid rate of growth with overshoot of response at higher intensities (>80 dB SPL). This very important work provided a framework for understanding how chronic physical noise damage affected cochlear physiology.

From around the 1980s, Salvi and colleagues also carried out many studies that were primarily aimed at looking at the effects of long term noise exposure (Salvi *et al* 1979a,b, 1983, Henderson *et al* 1983, Boettcher *et al* 1992). However, they are less directly applicable to the acute model described in this thesis. The authors tended to use lower frequency bandpass noise delivered over 5-15 days at about 95 dB SPL, and both TTS and PTS shifts were measured.

The finding of most likely relevance to this current study was the absence of apparent increase in suprathreshold CAP N_1 latency. This suggests two things; firstly noise damage in these recovered animals was not causing excessive or chronic excitotoxic neural damage. Secondly the conduction processes up to the point of noise damage had not been adversely affected (Salvi *et al*, 1979a,b).

Effects of noise on IHC and OHC - Because of the difficulty in recording *in vivo* from IHCs and OHCs, few reports of direct effects of noise on these cells have been made (Cody and Russell, 1988). These were not recorded after prolonged noise exposures, but instead were carried out using brief, repeated (225 ms) 12.5 kHz tones at 110 dB SPL. The authors found that IHC sensitivity and tuning were affected, recovering over 6-8 minutes post exposure. Most importantly, during noise exposure the IHC transfer function became more symmetrical and was therefore less able to generate a *dc* receptor potential. The OHCs also became acutely depolarised which would be expected to reduce their electromotility.

Whilst this study was very limited, it did show that the physiology of these two cells types was profoundly affected, independent of physical damage. This in particular would provide another contribution to NIHL.

Mismatch between noise frequency and tonotopic damage maxima (the 'half octave' shift) - A general feature reported by many workers is that following noise exposure, maximal damage along the BM does not occur at the frequency (or frequency band) of the noise stimulus (Henderson *et al*, 1983, Salvi *et al*, 1979, Boettcher *et al,* 1992). Instead, damage is spread over frequencies more basal to the stimulus frequency. This is typically reported to be maximal at about a half octave above the frequency (or frequencies) used to damage the BM.

This observation has been explained by adopting the features of non-linear models used to describe BM mechanics proposed by Davis (1983) Johnstone *et al* (1986) and Mountain (1986). In brief, this model explains the 'half octave' shift due to the cochlear amplifier actually operating maximally about half an octave up from the optimal point of vibration for the equivalent frequency point on the BM/Organ of Corti. Consequently, the centre frequency or frequencies of the noise insult as shown in figure 4.6, results in substantial physical damage at a half octave location above the insult.

Acute models of NIHL investigating physical versus excitotoxic damage - The physiological and anatomical studies reviewed above have been very important in increasing the understanding of the mechanisms of NIHL. However, few researchers have addressed the issue of what components of damage to the CN may have arisen due to a transient or permanent excitotoxic effect. This question, particularly with regards to pharmacology and anatomy, has been addressed by Puel (1995) Puel *et al* (1995, 1998) d'Aldin *et al* (1997) and Pujol and Puel (1999) as reviewed in Chapter 1.

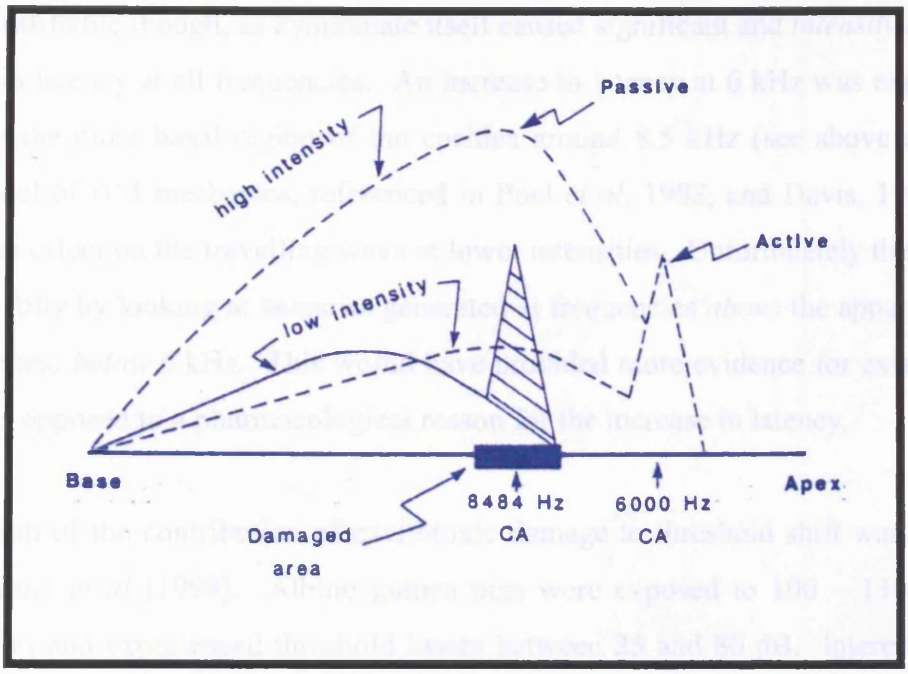


Figure 4.6. Schematic presentation of the effects of a 6 kHz, 95dB 15 minute noise exposure. This describes the biphasic responses seen after insult. Active processes involved at the lower sound intensities with the passive input resulting in the ½ octave shift theory causing damage at 8484 Hz (CAP latencies are increased at 6 kHz but are reportedly unaffected at 8 kHz due to the apparent lack of damage basal to this region). Taken from Puel *et al*, 1988.

Physiologically, only Puel *et al* (1988, 1998) appear to have specifically addressed the question of discriminating between physical and excitotoxic damage following acute noise exposure. In their first study, pigmented guinea pigs were exposed to a 6 kHz tone at 95 dB SPL for 15 minutes. Both CAP N_1 and SP threshold, amplitude and latencies were measured, along with CM at 6, 8.5 and 10 kHz. The authors also perfused cochleae with artificial perilymph in order to deliver kynurenate, a weak broad-spectrum GluR competitive inhibitor, with the aim of decreasing (and presumably ameliorating) excitotoxic effects in the cochlea.

Following noise insult, maximal effects were seen at 8.5 kHz (ie. about a half octave up from 6 kHz) and comprised elevation of threshold and depression of the lower intensity amplitude, paralleled by changes in SP ie up to about 80 dB SPL. No effect on CAP latency was seen. At 6 kHz, relatively small effects were seen on amplitude and SP, but significant *intensity dependent* effects on latency (ie increases of about 0.1-0.5ms) were observed up to 80 dB SPL. No protective effects of kynurenate were observed at any frequency. It was concluded from this that, at 8.5 kHz, the changes in CAP amplitude and SP were due to a reduction in output at the active amplifier (OHC) stage. If significant excitotoxic effects were observed the insult would also have been expected to reveal significant shifts in latency due to compromised CN function. This latter conclusion is

not fully justifiable though, as kynurenate itself caused significant and *intensity dependent* increases in latency at all frequencies. An increase in latency at 6 kHz was explained by damage to the more basal region of the cochlea around 8.5 kHz (see above citation of active model of BM mechanics, referenced in Puel *et al*, 1988, and Davis, 1983). This then had an effect on the travelling wave at lower intensities. Unfortunately this idea was not tested fully by looking at latencies generated at frequencies *above* the apparent region of damage and *below* 6 kHz. This would have provided more evidence for existence of a physical as opposed to a pharmacological reason for the increase in latency.

The question of the contribution of excitotoxic damage to threshold shift was addressed again by Puel *et al* (1998). Albino guinea pigs were exposed to 100 – 130 dB noise (6 kHz tone) and experienced threshold losses between 25 and 80 dB. Interestingly, the authors showed a frequency dependent result that was linked to the noise insult but rather disappointingly, N_1 latencies and amplitudes were not measured. After 100 dB the most affected frequency was 10 kHz, whereas at 130 dB, the most affected frequency was 16 kHz. Evidence from this work also showed that dendritic damage was only evident after 120 and 130 dB SPL along with some signs of damage to the outer row of the OHCs.

Evidence to link this with glutamate release was again provided by kynurenate (5mM concentration). Firstly, in the absence of kynurenate, peak threshold shifts of 60-80 dB were seen after 15 minutes exposure to 130 dB SPL. In the presence of kynurenate however, thresholds were reduced to 30-40 dB directly after perfusion. This suggested that about 40dB of the threshold shift was attributable to acute excitotoxic effects. Over 30 days partial recovery was seen, with peak PTS over 6-12 kHz of 15-20 dB. Interestingly, the authors also showed that with increasing intensity the maximal shift of threshold moved higher up the frequency range (16 kHz at 130 dB SPL) occurred. This was also accompanied by recruitment of frequencies below 6 kHz. At 130 dB SPL, all these frequencies also appeared to have an excitotoxic component to their threshold shift (figure 4.7). The authors concluded that excitotoxic damage was only significant at higher intensities and that synaptic repair (described elsewhere by Puel *et al* 1995) accounted for the TTS recovery seen over 30 days. They also proposed that the initial 50 dB or so of threshold elevation were due to non-excitotoxic damage.

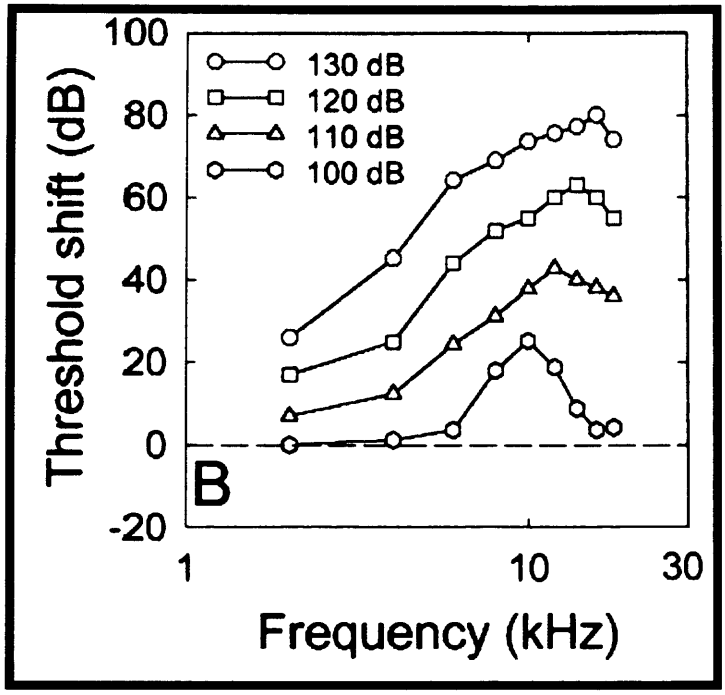


Figure 4.7. CAP threshold shift calculated as the difference from the recording before and 20 minutes after 6 kHz continuous tone exposure. Shown, are threshold shift recorded after 100 (empty hexagon), 110 (empty triangle), 120 (empty square), and 130 dB SPL (empty circle) exposure during 15 minutes. Taken from Puel *et al*, 1998.

4.7. The role of the of middle ear and efferent system in protecting against NIHL

The protective roles played by the middle ear muscles and the cochlear efferent system are reviewed by Henderson *et al* (1993). Whilst this area does not appear to have been systematically reviewed, the middle ear muscles in man do seem to provide some protection against TTS below about 4 kHz (Zakrisson *et al*, 1980). In experimental animals the position is less clear. In the cat, for example, Liberman (1992) showed that cutting both the middle ear muscles and the efferent supply to the cochlea did not provide a significant degree of protection against TTS in response to 6 kHz at 100 dB SPL for 10 minutes. So far, no studies demonstrating the protective role of the middle ear and efferent system against NIHL in the guinea pig have been carried out.

4.8. Physiological changes in the cochlea following hypoxia exposure

General hypoxia, or anoxia induced by short-term respiratory interventions, has often been employed to impair oxygenation of the inner ear in order to study its effects on the cochlea and CN physiology in both animals and humans (Evans, 1974, Gafni and Sohmer, 1976, Nuttall and Lawrence, 1980, Fechter *et al*, 1987 and 1988, Billett *et al*, 1989, Sohmer *et al*, 1989, Haupt *et al* 1993, Pujol *et al*, 1990). Interruption of cochlear blood supply not surprisingly results in a rapid and marked detrimental effect on various

cochlea potentials, highlighting the very high metabolic demand and turnover in the cochlea as well as an inability to retain oxygen reserves (Lamm and Arnold 1999, 2000). Hearing assessment in these studies following exposure has been made within minutes, several hours (Cycowicz *et al*, 1988) and up to six months (Jiang, 1995).

The studies briefly reviewed below summarise the findings most relevant to results presented in this thesis but they also reveal that most studies looking at the effects of hypoxia on the cochlea have used either total anoxia or low levels of inspired oxygen (10% and below). In terms of the levels of hypoxia employed, these studies can be further divided into those employing very low hypoxic/anoxic levels (0-5% O_2) low level hypoxia (5-10% O_2) and moderate hypoxia (10% O_2 and above).

4.8.1. Hypoxia studies using very low O_2 levels (0-5%)

The use of acute severe hypoxia lasting from 30 seconds to a few minutes, achieved by occlusion of cochlear blood supply or by asphyxia, revealed a clear pattern in collapse and recovery of gross cochlear potentials (Konishi *et al,* 1961, Fechter *et al,* 1987). Within seconds of asphyxia, the EP rapidly declined, followed by the CAP and the CM. After re-instigating normoxia, the EP quickly returned to a normal or near normal level, followed by CM and then more slowly the CAP.

Investigations using both gross cochlear electrodes and microelectrodes to record from IHCs further showed that IHC potentials were particularly vulnerable to acute asphyxia (Russell and Cowley, 1983, Brown *et al* 1983). The *dc* receptor potential (ie the potential generated in response to stimuli) was affected, following a similar time course to CAP deterioration and recovery. The IHC FTC also showed a loss of sensitivity and broadening of tuning. Russell and Cowley (1983) also showed that the IHC transfer function lost its asymmetry, suggesting that the mechanism responsible for IHC current rectification is very sensitive to an acute reduction in O_2.

At the level of the CN in both the cat and the guinea pig, acute asphyxia lasting between 2 to 5 minutes has been shown to dramatically increase the threshold of single nerve fibres. This was also accompanied by a rapid drop in the SR of nerve fibres, although recovery of SR was dissociated from recovery of fibre threshold, taking longer to recover to normal levels (Evans 1975, Manley and Robertson 1976).

These studies underscore the vulnerability of the cochlea to the very short-term cessation of O_2. Importantly, they also point to the IHC and CN as being particularly vulnerable to very low levels of O_2. This is also borne out by the comparatively very high metabolic demand of these sites especially when subject to acoustic stimulus, as shown in figure 4.5 (Ryan, 1988).

4.8.2. Hypoxia studies using low O_2 levels (5-10%)

Studies using 5-10% hypoxia have typically been conducted over a longer time period to observe physiological effects. Unfortunately, the results from these studies may have been confounded in part by only using band limited 'click' stimuli (typically up to 8-10 kHz) which would tonotopically limit the evoked response. This is important as the lower frequencies in the cochlea are less susceptible to insult (Pickles, 1991, Haupt *et al*, 1993).

Haupt *et al* (1993) exposed guinea pigs to 8% oxygen in nitrogen for one hour and revealed a slight drop in heart rate and a statistically significant drop in blood pressure over time. This caused an 80 to 85% depression in the CM, CAP and BAER amplitude after 30 minutes, although the decreases were not observed until 20 minutes after exposure. No significant increases in wave latencies were recorded and thresholds were not apparently measured. Re-ventilation with normal air for 30 minutes revealed either a slight overshoot or a return to normal amplitude values. Interestingly, 10% oxygen for one hour was reported as showing no significant functional loss. An important observation from this work was that mean PO_2 in the basal scala tympani perilymph during hypoxia showed a dramatic drop within 5 minutes (70% of the initial value) reaching a maximum with 10 minutes. Between 10 and 30 minutes no appreciable differences were then noted. This was important as it showed that the first 15 minutes of recording were important in monitoring changes in cochlear function.

In the cat, Gafni and Sohmer (1976) measured the EP after varying inspired O_2 between 2-10%. Below 10% there were marked idiosyncratic drops in EP, but remained stable at 10%. Also using the cat, Sohmer *et al* (1989) measured BAER thresholds exposed to 4-10% oxygen in nitrogen (exposure to 4% oxygen was not viable due to overt physiological changes in the animal). They reported no change in response at 10% O_2, but after 20 minutes at 6%, an increase of 15 dB in threshold with a depression in BAER amplitude were seen combined with a 70% drop in perilymphatic O_2. However, these changes were accompanied by a statistically significant drop in blood pressure, which

could have further contributed to deterioration in function secondary to hypoxia. Normal function was restored after 30 minutes re-ventilation.

4.8.3. Hypoxia studies using moderate O_2 levels (10-16%)

Fewer studies appear to have been carried out over this range, but they have shed some light on the vulnerability of the cochlea to modest reductions in inspired O_2. Nuttall and Lawrence (1980) found that in the guinea pig, the endolymphatic PO_2 level remained stable down to about 16%. The authors also found that cochlear potentials remained relatively unaffected down to about 12% but below this they began to deteriorate (figure 4.8).

From this literature review it is clear that marked hypoxia leads to deficits in cochlear performance. The few studies concerned with marginal O_2 levels suggest that levels down to about 10-12% are adequate to maintain basal function. Importantly, for this study the published data support the idea of a marginal hypoxia of about 12% in combination with noise. Whilst levels of 12% oxygen and above may not cause any changes in cochlear function during the first 15 minutes of exposure, synergism with noise is likely to increase the metabolic demand on the cochlea thereby affecting cochlear function.

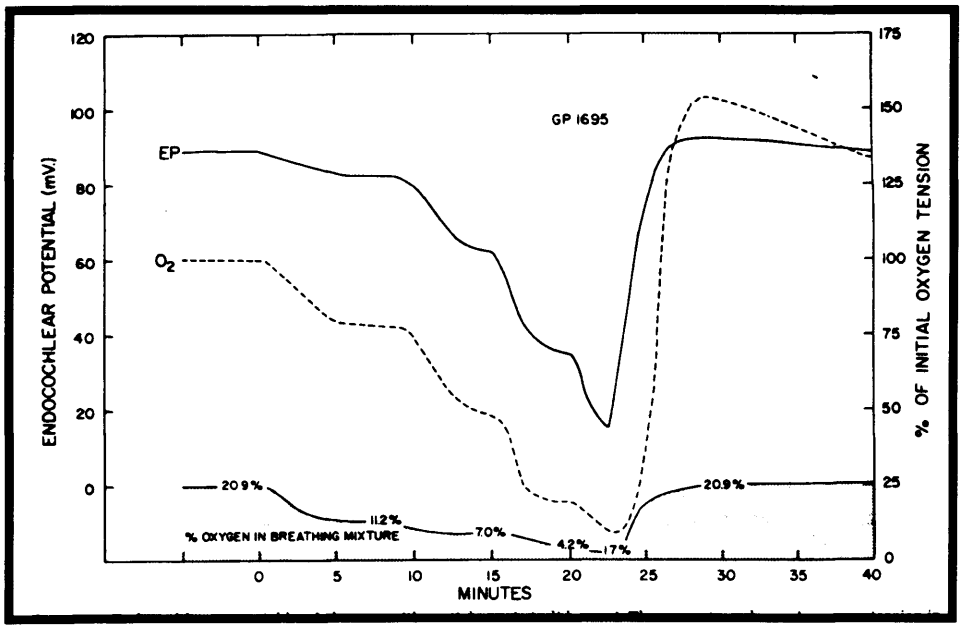


Figure 4.8. Endocochlear potential level changes with partial anoxia (solid line). Percentage change in oxygen tension in the scala media (dotted line is also shown). The shaded area at the bottom represents the percentage of oxygen in the breathing mixture. Note the small changes observed after exposure to 12% oxygen. Taken from Nuttall and Lawrence, 1980.

4.9. The effects of Noise/Hypoxia on cochlear function

A review of the literature has revealed very few systematic studies looking at the synergistic effects of noise and reduced oxygen on cochlear function. Clear synergy between noise and carbon monoxide (CO) induced hypoxia was initially demonstrated by Young *et al* (1987) in pigmented rats. However, most studies in this area have been carried out by Fechter and colleagues, using carbon monoxide (CO) as the hypoxic agent (Young *et al*, 1987, Fechter *et al*, 1988 & 2000, Chen *et al*, 1999 & 2001, Rao and Fechter, 2000).

The startle response technique was employed by Young *et al* (1987) at 10 and 40 kHz, following exposure to 2 hours of 4-8 kHz bandpass noise (approximately 105 dB SPL). A 90 minute pre-CO exposure period (1200ppm) was used to allow binding with haemoglobin prior to any noise insult. Subsequent CO exposure was the given either alone, or in combination with the noise insult. Exposure to CO alone did not result in any acute threshold shift, but noise alone caused an approximate shift of about 20 dB at 10 kHz and 15 dB at 40 kHz. Exposure to noise/CO however, revealed greater acute threshold changes. At 40 kHz, acute increases were comparatively greater than at 10 kHz being up by about 35 dB. Importantly, these results also uncovered a very clear tonotopic susceptibility to noise/hypoxia, causing more severe damage at frequencies well above the 4-8 kHz frequency band of the noise insult. This in turn illustrates the *integral* involvement of the cochlea as a whole organ in processing incoming stimuli.

Severity of damage is dependent on noise exposure and level of CO - Fechter *et al* (1988) extended this work by looking at chronic cochlear deficit measured electrophysiologically, including more detailed frequency measurements between 2.5 to 40 kHz. Both noise and CO exposures were employed as before with thresholds being measured 6 to 8 weeks post exposure. Again, no effect on threshold was observed in the CO group, but noise exposure resulted in an approximate 10 dB shift and tended to be greatest in the 10 to 14 kHz region. Surface preparation microscopy revealed basal OHC loss.

Exposure to both noise and CO, however, revealed large threshold changes especially over 10 and 40 kHz (ranging from 40 to 60 dB) providing clear evidence of PTS. Additionally, microscopy showed there was extensive IHC and OHCs damage after

noise/CO exposure. From the basal end, this extended down 15% and up to 60% of the length of the cochlea for IHCs and OHCs respectively. Using this experimental paradigm, further studies by Fechter and colleagues were conducted varying both noise duration and level of CO (Chen *et al* 1999 Fechter *et al* (2000). As expected, severity of functional deficit and hair cell loss was shown to be dependent on noise duration and level of pre-noise exposure to CO. The effects of varying CO concentration for a given noise exposure are shown in Figure 4.9. An additional observation is that carboxyhaemoglobin levels of up to 56% were achieved in these studies. The work by Fechter and colleagues clearly demonstrated synergy between noise and hypoxia. It also showed that, in the rat, it leads to both TTS and PTS, and a very marked tonotopic recruitment effect on threshold elevation at both higher and lower frequencies. They also demonstrated a clear dependence on noise duration and hypoxia level. One limitation of these NH studies is that no measures of suprathreshold gross CAP input/output function were carried out, which was also a limitation of much of the noise alone literature. This, in particular, was one area which the studies presented in this chapter has attempted to cover in more detail.

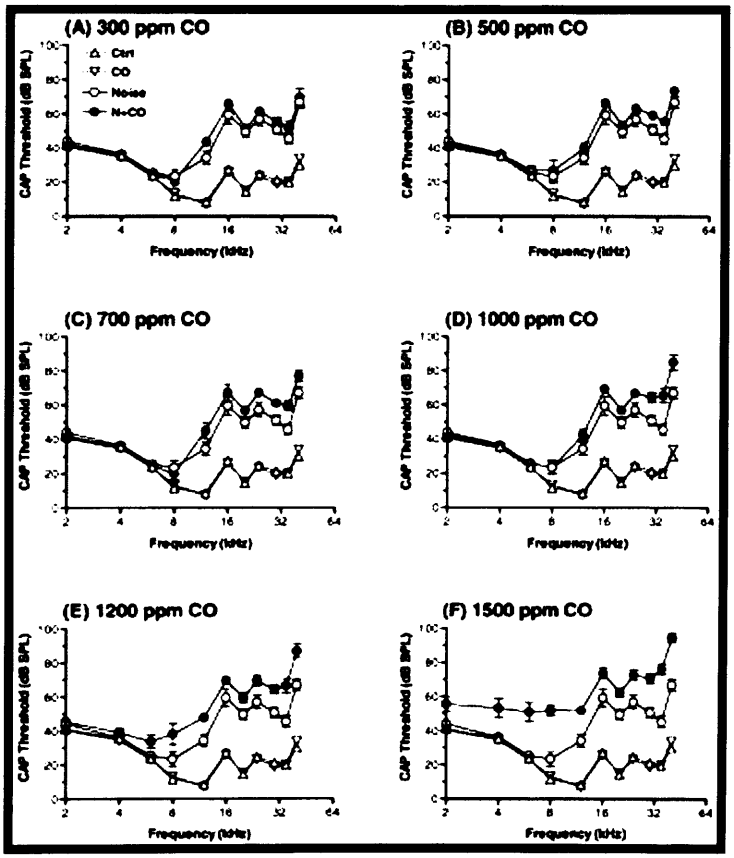


Figure 4.9. Pure tone auditory thresholds for subjects exposed to a fixed noise level (100 dB, 13.6 kHz centred for 8 hours) alone or in combination with CO at concentrations of 300–1500 ppm (A to F). All thresholds were recorded 4 weeks following experimental exposures. Taken from Fechter *et al*, 2000.

4.10. Experimental methods

The study comprised four treatment groups (28 animals) as shown in Table 4.1. Sedation was as defined in Regimen 3, Chapter 2, section 2.4 and electrophysiological recordings in sections 2.9 to 2.11.

Briefly, immediately after surgery, CAP (8, 16, 24 and 30 kHz) and ESAC/EDAC measurements were taken on at least four separate occasions, usually at least 5 minutes apart, including 3 measurements taken during a 15 minute artificial perilymph pretreatment period. Animals were then exposed to either normal air for up to 60 minutes (control) or 15 minutes of noise (approximately 100 dB SPL) 12% oxygen or a combination of both, followed by a very short recovery period. Control CAP/ESAC measurements were performed at 15 minute intervals during the 60 minute time period whilst in treated animals, recordings were performed at 5 minute intervals during and immediately after the 15 minute exposure period. Control animals were reduced to 6 for the purposes of ESAC recordings due to constraints on the experimental design. Table 4.2 below shows dB values at 0, 20, 40 and 60dB attenuation for all four frequencies.

Table 4.1. Study Design

Group Number	Treatment	Number of animals
1	Control (artificial perilymph)	7
2	Noise (100 dB)	7
3	12% Hypoxia (12% oxygen)	4
4	Noise (100 dB) and 12% hypoxia	10

Table 4.2. dB values for all levels of attenuation

	Frequency (kHz) and dB values following attenuation			
dB attenuation	**8**	**16**	**24**	**30**
0	92	92	82.5	84
20	72	72	62.5	64
40	52	52	42.5	44
60	32	32	22.5	24

4.11. Results

As for Chapter 3, because of unequal numbers of animals across treatment groups the results were principally analysed first using repeated measures one way ANOVA to examine the effects of insult over time *within* a treatment group. Differences *between* treatment groups (especially noise and noise/hypoxia) were then investigated again using one way ANOVA. This involved consideration of the effects of time/insult on CAP threshold, amplitude and latency and ESAC power. Detailed analysis of these parameters helped to define any synergistic effects of noise and 12% hypoxia on cochlear function. The within group results are summarised in Appendix 1.

4.11.1. CAP threshold changes

Time control data - Time control CAP thresholds over 60 minutes revealed very stable recordings (figure 4.10). Significantly, recordings made at –15 minutes (immediately after surgery) were comparable to those made at the beginning of artificial perilymph application (0 minutes or T_0) indicating no effect of fluid application on cochlear sensitivity at any frequency.

Over the 60 minutes recording period, 16 and 24 kHz recorded the best mean threshold values (74.9 ±0.9 and 76.6 dB SPL ± 5.0 respectively) whereas 8 and 30 kHz were consistently lower (64.4±4.6 and 61.9±9.2 dB SPL). The larger standard error data recorded for 30 kHz was due to two animals that showed lower than normal threshold values at this frequency. These control results showed that consistently stable recordings could be made in the presence of artificial perilymph over 1 hour.

The effects of 15 minutes 12% hypoxia exposure - Exposure to 12% hypoxia did not produce any significant changes in threshold after 15 minutes (figure 4.11). The most marked change was a slight but not significant decrease in mean threshold from T_0 observed at 8 kHz of 2.25 dB SPL. No other frequencies were affected.

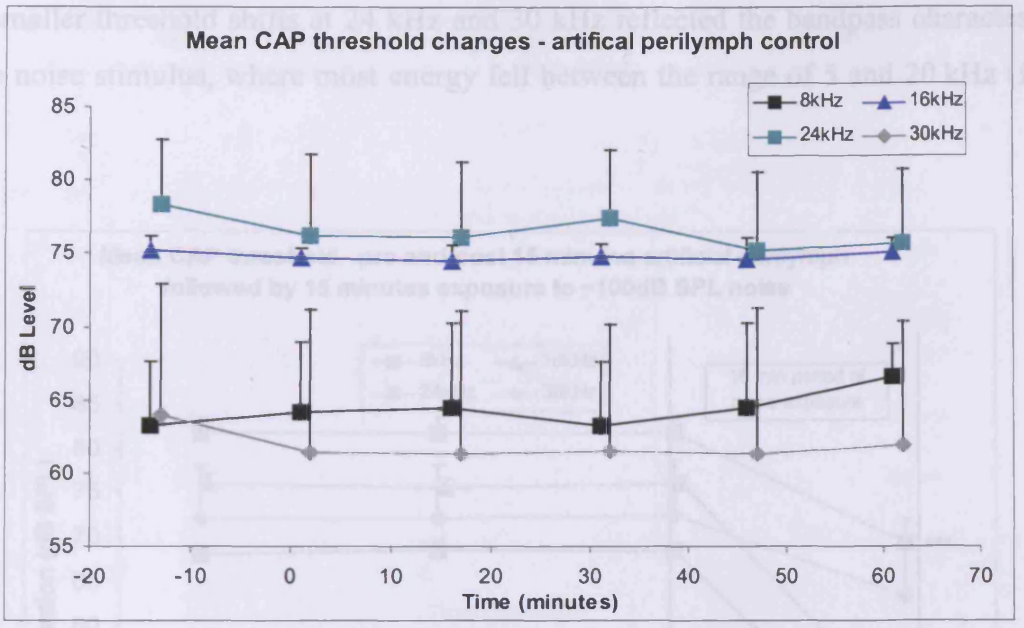


Figure 4.10. Mean artificial perilymph time control CAP threshold changes over 60 minutes (n=7). CAP thresholds remained stable over this recording period.

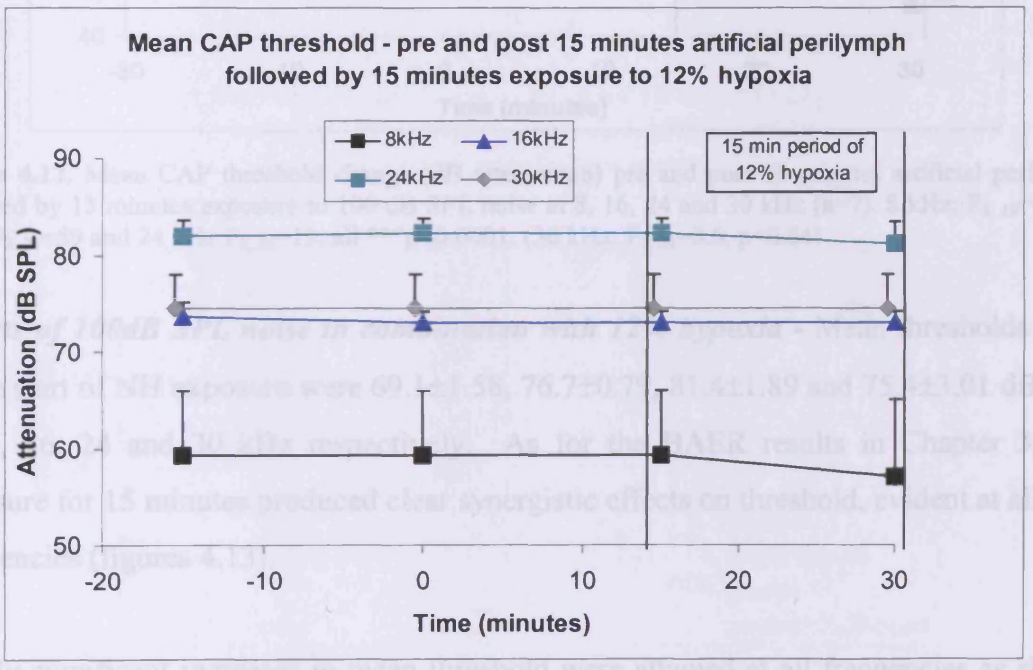


Figure 4.11. Mean CAP threshold change (dB attenuation) pre and post 15 minutes artificial perilymph followed by 15 minutes exposure to 12% hypoxia at 8, 16, 24 and 30 kHz (n=4). No CAP thresholds were significantly affected by this duration of hypoxia exposure.

The effects of 15 minute 100 dB SPL noise exposure - Mean thresholds prior to the start of noise exposure were 68.4±0.98, 76.0±0.93, 81.9±1.55 and 72.4±6.12 dB SPL for 8, 16, 24 and 30 kHz respectively. When compared to pre-artificial perilymph data, exposure to 100 dB SPL for 15 minutes produced highly significant increases in threshold at 8 16 and 24 kHz. Figures 4.12 and 4.14 show the greatest mean threshold shift of 26 dB was at 16 kHz, closely followed by a similar shift of 25.2 dB at 8 kHz and then 12.4 dB at 24 kHz.

The smaller threshold shifts at 24 kHz and 30 kHz reflected the bandpass characteristics of the noise stimulus, where most energy fell between the range of 5 and 20 kHz (figure 4.1).

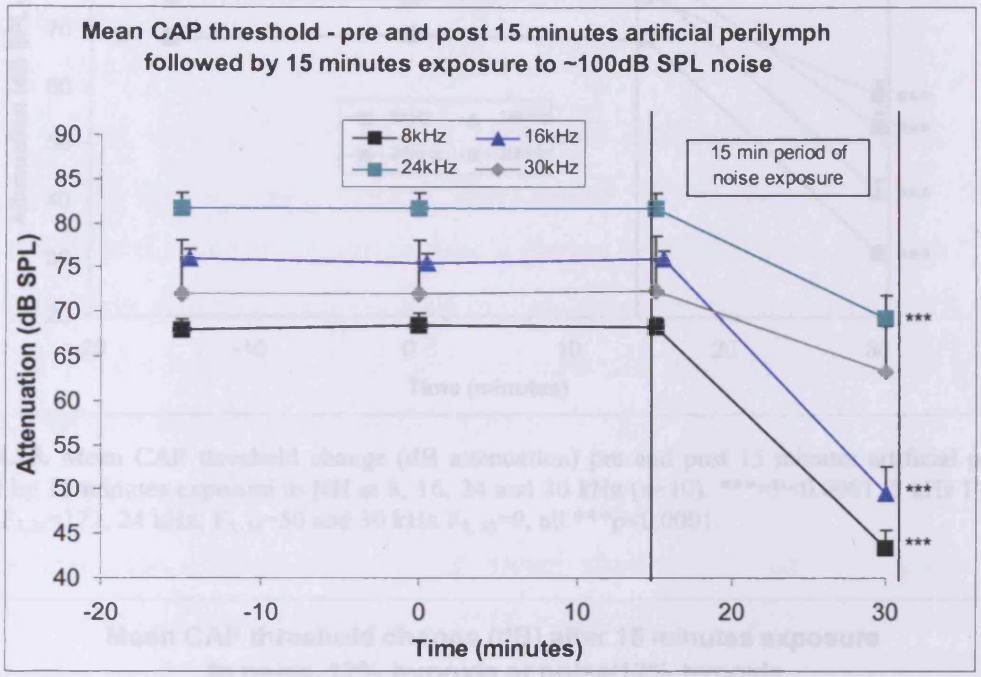


Figure 4.12. Mean CAP threshold change (dB attenuation) pre and post 15 minutes artificial perilymph followed by 15 minutes exposure to 100 dB SPL noise at 8, 16, 24 and 30 kHz (n=7). 8 kHz; $F_{3, 19}$=69, 16 kHz; $F_{3, 24}$=59 and 24 kHz $F_{3, 24}$=12, all ***p<0.0001. (30 kHz; $F_{3, 24}$=0.6, p<0.64)

Effects of 100dB SPL noise in combination with 12% hypoxia - Mean thresholds prior to the start of NH exposure were 69.1±1.58, 76.7±0.79, 81.4±1.89 and 75.4±3.01 dB SPL for 8, 16, 24 and 30 kHz respectively. As for the BAER results in Chapter 3, NH exposure for 15 minutes produced clear synergistic effects on threshold, evident at all four frequencies (figures 4.13).

Highly significant increases in mean threshold were attained at all frequencies as shown in figure 4.14, with the greatest threshold shift of 37.5 dB at 8 kHz, closely followed by 16 kHz (35.7 dB) and then 24 kHz (28.6 dB). The pattern of threshold elevation again followed the band pass characteristic of the noise stimuli, although statistical significance was attained at 30 kHz (16.7 dB). This threshold change was approximately double that observed with noise alone (8.7 dB) providing further evidence of synergy with 12% hypoxia affecting cochlear function compared to noise exposure alone.

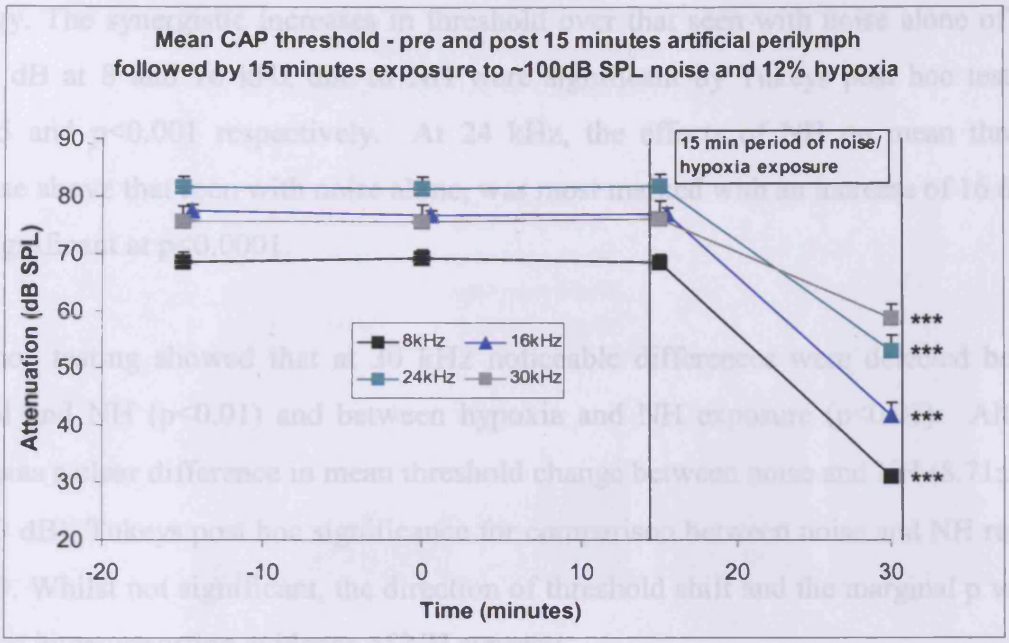


Figure 4.13. Mean CAP threshold change (dB attenuation) pre and post 15 minutes artificial perilymph followed by 15 minutes exposure to NH at 8, 16, 24 and 30 kHz (n=10). ***=P<0.0001. 8 kHz $F_{3,36}$=146, 16 kHz; $F_{3,36}$=172, 24 kHz; $F_{3,36}$=50 and 30 kHz $F_{3,32}$=9, all ***p<0.0001.

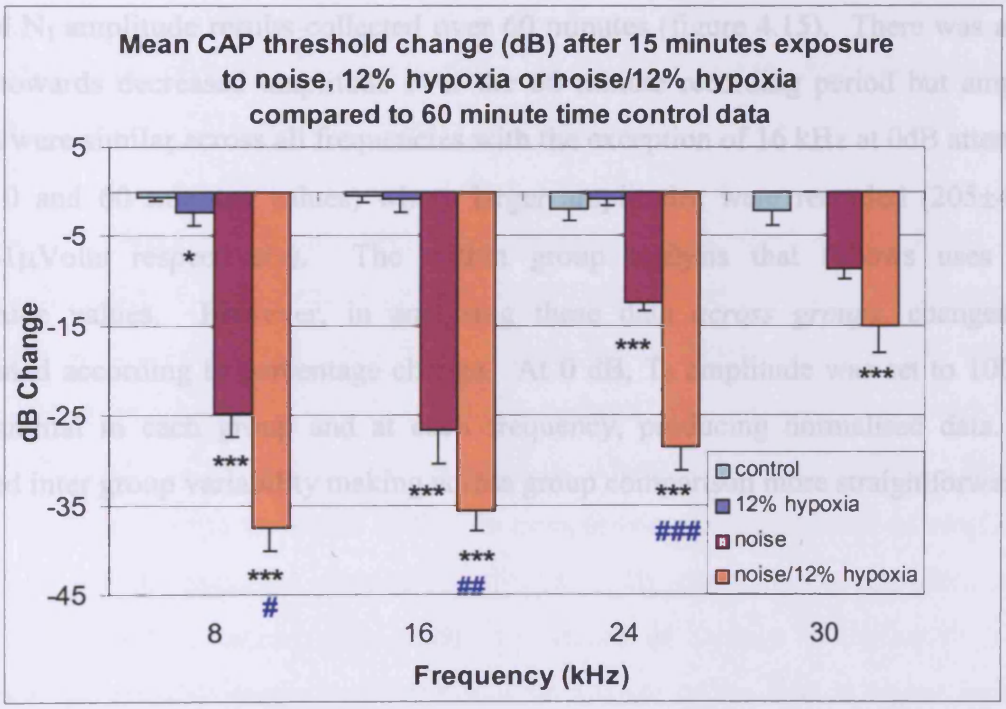


Figure 4.14. A summary of the across group mean CAP threshold changes after 15 minutes exposure to artificial perilymph, 12% hypoxia, noise or NH (n=7, 4, 7 and 10 respectively). $F_{3,23}$=8.77 p<0.0001, $F_{3,25}$=51.32 p<0.001, $F_{3,25}$=56.08 and $F_{3,24}$=69.59 p<0.0001 (30, 24, 16 and 8 kHz respectively). *=p<0.05, ***=p<0.001 control versus hypoxia, noise or NH (#, ##, ### p<0.05, <0.001 and <0.0001 noise versus NH). Synergy was seen at 8, 16 and 24 kHz with the greatest effect observed at 24 kHz.

Across group comparison – ANOVA returned highly significant changes in CAP threshold at all frequencies across all groups. As was expected from the *within* group analysis, no significant differences were noted between the control and hypoxia groups. Noise vs NH post hoc group comparisons, however, were of most interest with respect to

synergy. The synergistic increases in threshold over that seen with noise alone of about 10-12 dB at 8 and 16 kHz due to NH were significant by Tukeys post hoc testing at $p<0.05$ and $p<0.001$ respectively. At 24 kHz, the effects of NH on mean threshold increase above that seen with noise alone, was most marked with an increase of 16 dB and was significant at $p<0.0001$.

Post hoc testing showed that at 30 kHz noticeable differences were detected between control and NH ($p<0.01$) and between hypoxia and NH exposure ($p<0.01$). Although there was a clear difference in mean threshold change between noise and NH (8.71±1.1 vs 15.0±3 dB), Tukeys post hoc significance for comparison between noise and NH returned $p=0.09$. Whilst not significant, the direction of threshold shift and the marginal p value is taken as intersupporting evidence of NH synergy.

4.11.2. CAP N_1 amplitude data

Time control data - There were no significant changes in mean artificial perilymph control N_1 amplitude results collected over 60 minutes (figure 4.15). There was a slight trend towards decreased amplitude over the 60 minute recording period but amplitude values were similar across all frequencies with the exception of 16 kHz at 0dB attenuation (both 0 and 60 minutes values) where larger amplitudes were recorded (205±41 and 172±41μVolts respectively). The within group analysis that follows uses actual amplitude values. However, in analysing these data *across groups*, changes were calculated according to percentage change. At 0 dB, T_0 amplitude was set to 100% for each animal in each group and at each frequency, producing normalised data. This reduced inter group variability making across group comparison more straightforward.

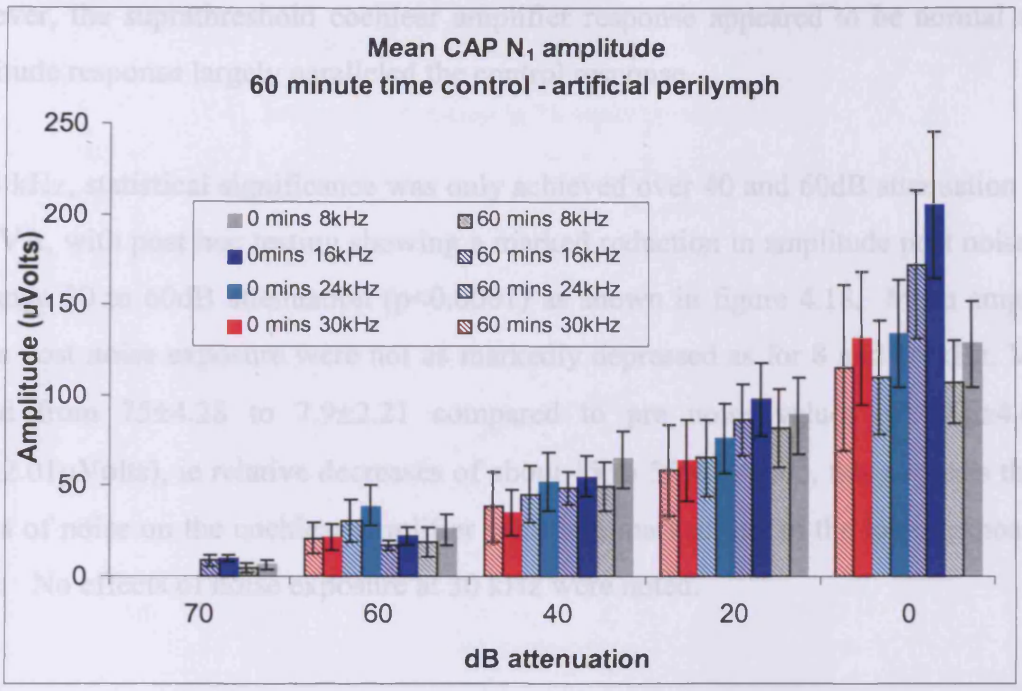


Figure 4.15. Mean artificial perilymph control CAP N_1 amplitude changes over 60 minutes (n=7). Diagonal line bars represent 60 minute data. Good stability over time was achieved with greater sensitivity for 16 kHz at 0dB attenuation compared to the other frequencies.

Exposure to 12% Hypoxia for 15 minutes – No effects on wave amplitude were noted at any frequency after 12% hypoxia exposure for 15 minutes.

Exposure to Noise for 15 minutes - No apparent effect of noise was noted at 0dB attenuation, 8 kHz. However, large and significant decreases in amplitude were noted over time at 20 and 40dB attenuation (figure 4.16). Mean values at 20 and 40dB attenuation were 19.4±4.4 and 2.7±1.1μVolts respectively compared to pre noise values of 52.3±7.6 and 39.2±4.1μVolts, representing decreases of between 60-90%. Results at 60dB are absent due to threshold shifts. In comparison with the effects on amplitude at 20 and 40 dB, the apparent absence of effect at 0 dB attenuation is not taken as non-pathophysiological. Instead, it is taken as evidence of damage to the active cochlear process that normally damps down the passive growth of the BM at higher intensities (Kiang *et al*, 1986 Kossl and Russell, 1992).

At 16 kHz, highly significant decreases in mean amplitude over 0 to 40 dB attenuation ranged from 75.7±3.91 to 8.4±0.74 compared to pre noise values of 132.7± 6.19 to 16.4±1.11μVolts (figure 4.17). These results suggest that noise affected the most sensitive part of the cochlear amplifier, as also evidenced by the threshold increase.

However, the suprathreshold cochlear amplifier response appeared to be normal as the amplitude response largely paralleled the control response.

At 24 kHz, statistical significance was only achieved over 40 and 60dB attenuation using ANOVA, with post hoc testing showing a marked reduction in amplitude post noise over the range 20 to 60dB attenuation ($p<0.0001$) as shown in figure 4.18. Mean amplitude values post noise exposure were not as markedly depressed as for 8 and 16 kHz. Values ranged from 75±4.28 to 7.9±2.21 compared to pre noise values of 90.6±4.04 to 19.1±2.01μVolts), ie relative decreases of about 15 to 55%. Again, this suggests that the effects of noise on the cochlear amplifier were less marked due to the lower exposure to insult. No effects of noise exposure at 30 kHz were noted.

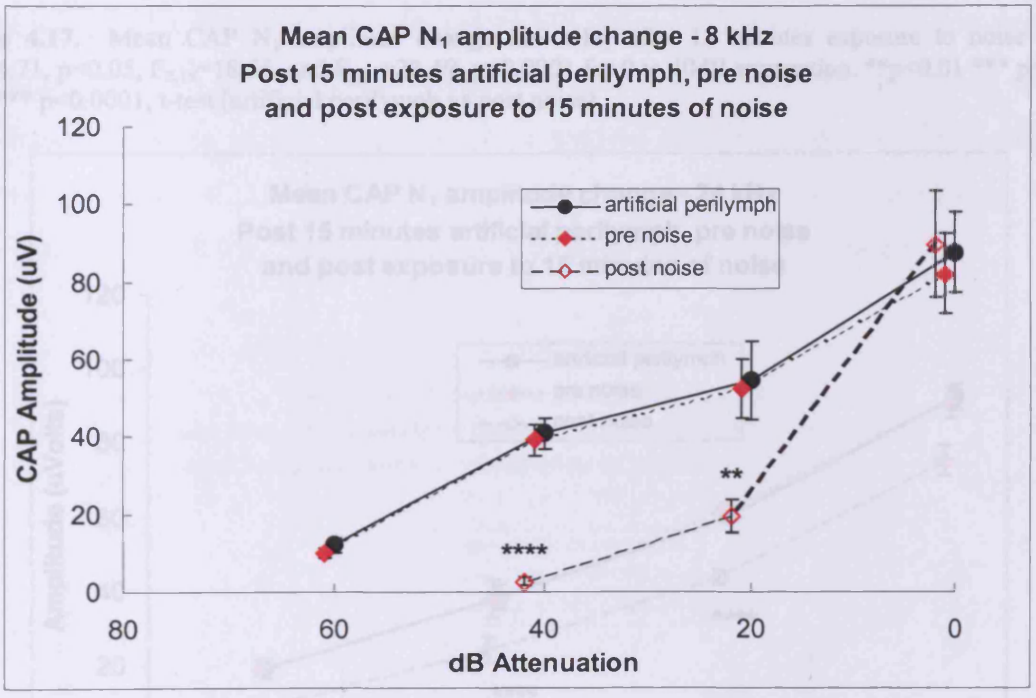


Figure 4.16. Mean CAP N_1 amplitude change (8 kHz) after 15 minutes exposure to noise (n=7). $F_{2,18}$=61.4, $p<0.01$ and $F_{2,15}$=39.95, $p<0.0001$ for 20 and 40dB attenuation. **p<0.01 and **** p<0.0001, t-test (artificial perilymph vs post noise). Note the marked depression of amplitude that then returns to control values at 0 dB attenuation following noise exposure.

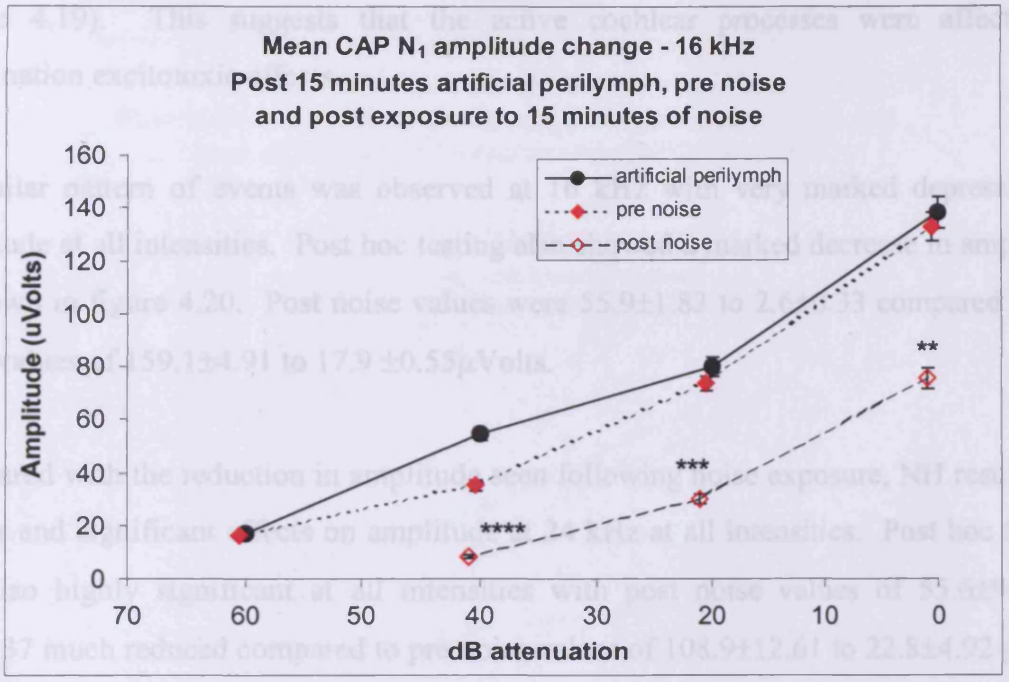


Figure 4.17. Mean CAP N_1 amplitude change (16 kHz) after 15 minutes exposure to noise (n=7). $F_{2,18}$=4.71, p<0.05, $F_{2,18}$=18.56, and $F_{2,18}$=23.49, p<0.0001 for 0 to 40dB attenuation. **p<0.01 *** p<0.001 and **** p<0.0001, t-test (artificial perilymph vs post noise).

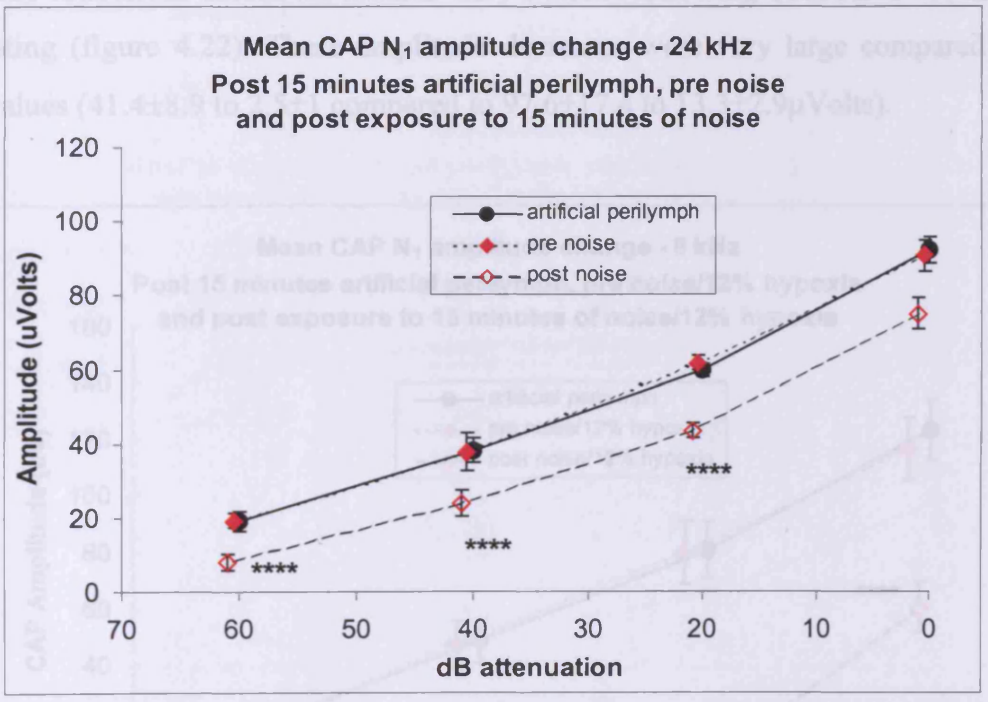


Figure 4.18. Mean CAP N_1 amplitude change (24 kHz) after 15 minutes exposure to noise (n=7). $F_{2,18}$=4.14, p<0.05 and $F_{2,18}$=8.18, p<0.01, ANOVA for 40 and 60dB attenuation. ****P<0.0001, t-test (artificial perilymph vs post noise).

Exposure to Noise/12% hypoxia (NH) – Figure 4.19 shows there were very large, significant decreases in amplitude at 8 kHz for 0 and 20dB attenuation following NH exposure. Post hoc testing revealed significance (p<0.0001) with mean values of 59.8±8.8 8.7±2μVolts compared to pre noise values of 116.3±11.1 and 79.6±11.4μVolts

(figure 4.19). This suggests that the active cochlear processes were affected in combination excitotoxic effects.

A similar pattern of events was observed at 16 kHz with very marked depression in amplitude at all intensities. Post hoc testing also showed a marked decrease in amplitude as shown in figure 4.20. Post noise values were 55.9±1.83 to 2.6±0.33 compared to pre noise values of 159.1±4.91 to 17.9 ±0.55μVolts.

Compared with the reduction in amplitude seen following noise exposure, NH resulted in greater and significant effects on amplitude at 24 kHz at all intensities. Post hoc testing was also highly significant at all intensities with post noise values of 55.6±9.17 to 0.5±0.37 much reduced compared to pre noise values of 108.9±12.61 to 22.8±4.92 μVolts (figure 4.21). The most marked evidence of NH synergy on amplitude was demonstrated at 30 kHz, where no effects of noise alone over time were noted. Significant and marked amplitude reductions across all stimuli were evident following both ANOVA and post hoc testing (figure 4.22). These amplitude decreases were very large compared to pre noise values (41.4±8.9 to 2.5±1 compared to 97.6±17.4 to 13.3±2.9μVolts).

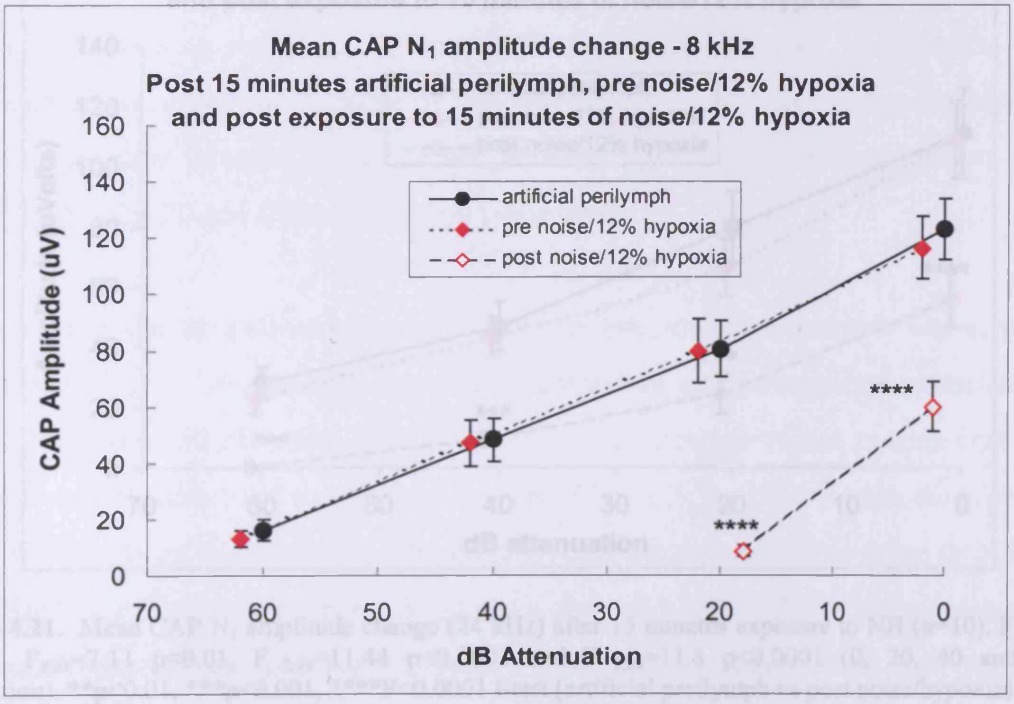


Figure 4.19. Mean CAP N_1 amplitude change (8 kHz) after 15 minutes exposure to NH (n=10). $F_{2,23}$=11.69 and $F_{2,22}$=25.64, p<0.0001, for 0 and 20dB attenuation. ****p<0.0001 t-test (artificial perilymph vs post noise/hypoxia).

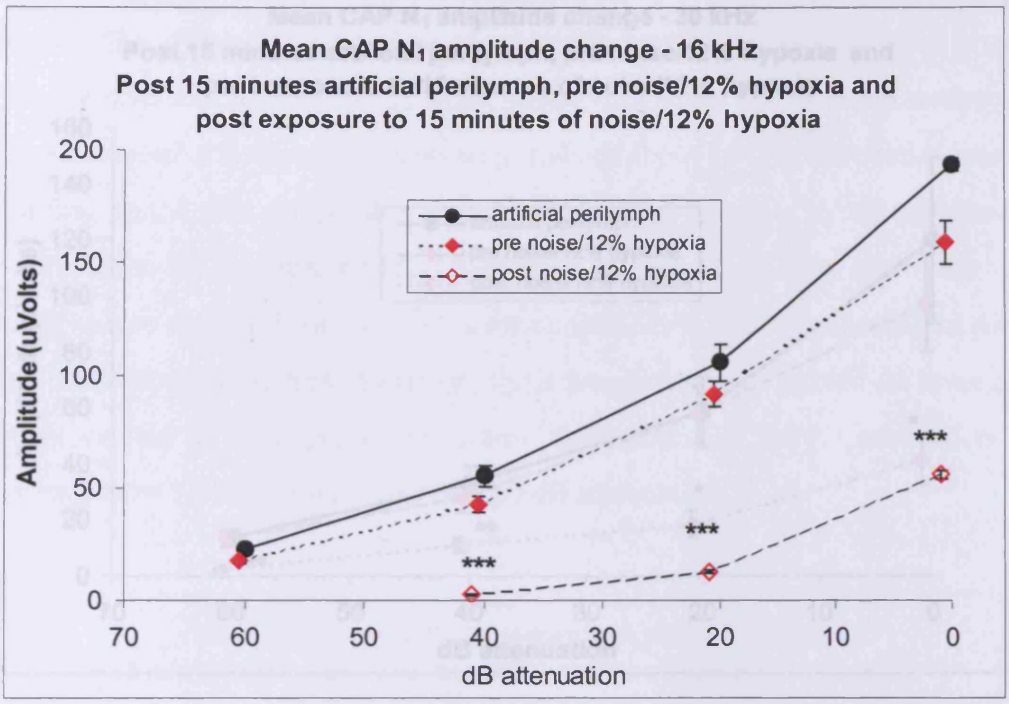


Figure 4.20. Mean CAP N_1 amplitude change (16 kHz) after 15 minutes exposure to NH (n=10). $F_{2,23}$=14.95, $F_{2,22}$=15.42 and $F_{2,23}$=18.58, p<0.0001 (0, 20 and 40dB attenuation). ***P<0.001, t-test (artificial perilymph vs post noise/hypoxia).

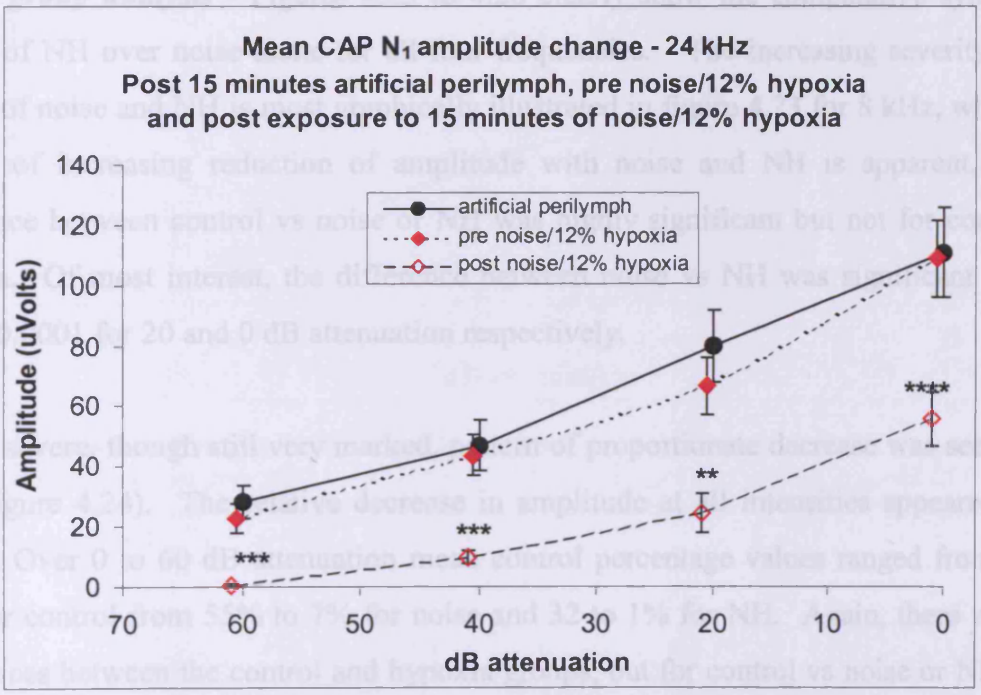


Figure 4.21. Mean CAP N_1 amplitude change (24 kHz) after 15 minutes exposure to NH (n=10). $F_{2,22}$=6.8 p<0.01, $F_{2,23}$=7.11 p<0.01, $F_{2,24}$=11.44 p<0.0001 and $F_{2,24}$=11.8 p<0.0001 (0, 20, 40 and 60dB attenuation). **p<0.01, ***p<0.001, ****P<0.0001 t-test (artificial perilymph vs post noise/hypoxia).

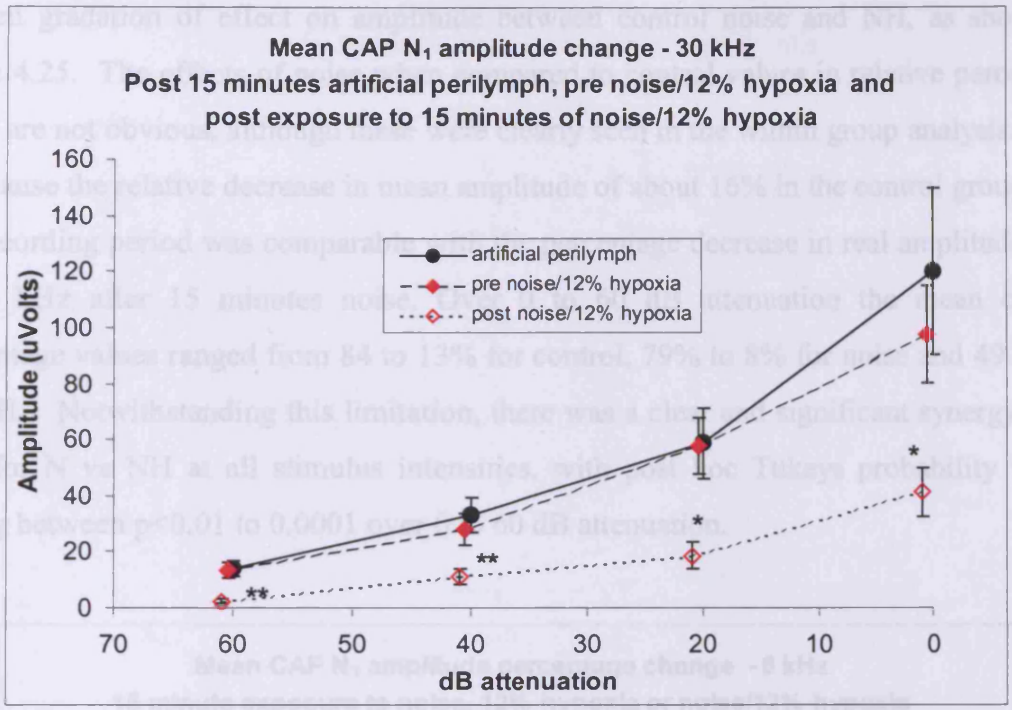


Figure 4.22. Mean CAP N_1 amplitude change (30 kHz) after 15 minutes exposure to NH (n=10). $F_{2,22}$=4.26 $p<0.05$, $F_{2,21}$=5.09 $p<0.05$, $F_{2,20}$=5.42 $p<0.05$ and $F_{2,18}$=8.98 $p<0.01$ (0, 20, 40 and 60dB attenuation). *$p<0.05$, **$p<0.01$, t-test (artificial perilymph vs post noise/hypoxia).

Across group analysis - Figures 4.23 to 4.26 clearly show the comparative synergistic effects of NH over noise alone for all four frequencies. The increasing severity of the effects of noise and NH is most graphically illustrated in figure 4.23 for 8 kHz, where the pattern of increasing reduction of amplitude with noise and NH is apparent. The difference between control vs noise or NH was highly significant but not for control vs hypoxia. Of most interest, the difference between noise vs NH was significant $p<0.01$ and $p<0.0001$ for 20 and 0 dB attenuation respectively.

A less severe, though still very marked, pattern of proportionate decrease was seen at 16 kHz (figure 4.24). The relative decrease in amplitude at all intensities appeared more graded. Over 0 to 60 dB attenuation mean control percentage values ranged from 86 to 11% for control from 55% to 7% for noise and 32 to 1% for NH. Again, there were no differences between the control and hypoxia groups, but for control vs noise or NH there were highly significant differences ($p<0.0001$). Specifically, the differences in percentage amplitude reduction for noise vs NH over 0 to 40 dB attenuation were significant over $p<0.05$ to 0.001.

At 24 and 30 kHz no major changes were seen after 12% hypoxia or noise exposure, but NH showed marked synergism across all frequencies. For 24 kHz, there was a less

marked gradation of effect on amplitude between control noise and NH, as shown in figure 4.25. The effects of noise when compared to control values in relative percentage terms are not obvious, although these were clearly seen in the within group analysis. This is because the relative decrease in mean amplitude of about 16% in the control group over the recording period was comparable with the percentage decrease in real amplitude seen at 24 kHz after 15 minutes noise. Over 0 to 60 dB attenuation the mean control percentage values ranged from 84 to 13% for control, 79% to 8% for noise and 49 to 6% for NH. Notwithstanding this limitation, there was a clear and significant synergy at 24 kHz for N vs NH at all stimulus intensities, with post hoc Tukeys probability values falling between $p<0.01$ to 0.0001 over 0 to 60 dB attenuation.

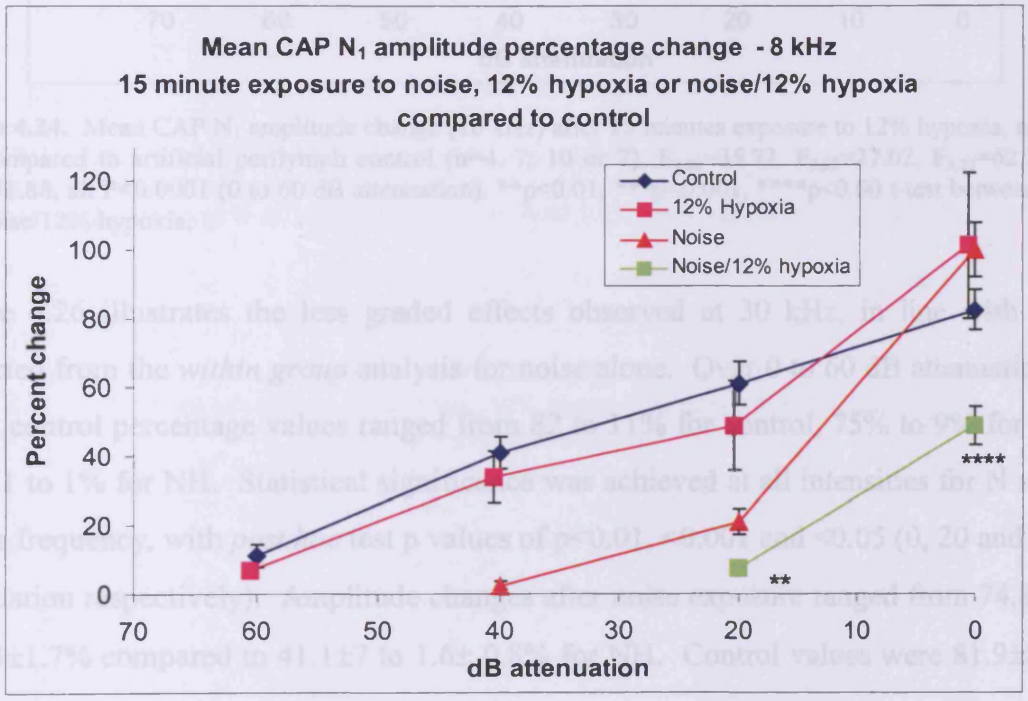


Figure 4.23. Mean CAP N_1 amplitude change (8 kHz) after 15 minutes exposure to 12% hypoxia, noise or NH compared to artificial perilymph control (n=4, 7, 10 or 7). $F_{3,22}=8.52$ $P<0.001$, $F_{3,22}=22.65$ $P<0.0001$, $F_{3,21}=49.37$ $P<0.0001$ and $F_{3,21}=14.26$ $P<0.001$ (0 to 60 dB attenuation). **p<0.01, ****P<0.0001, t-test between noise and NH.

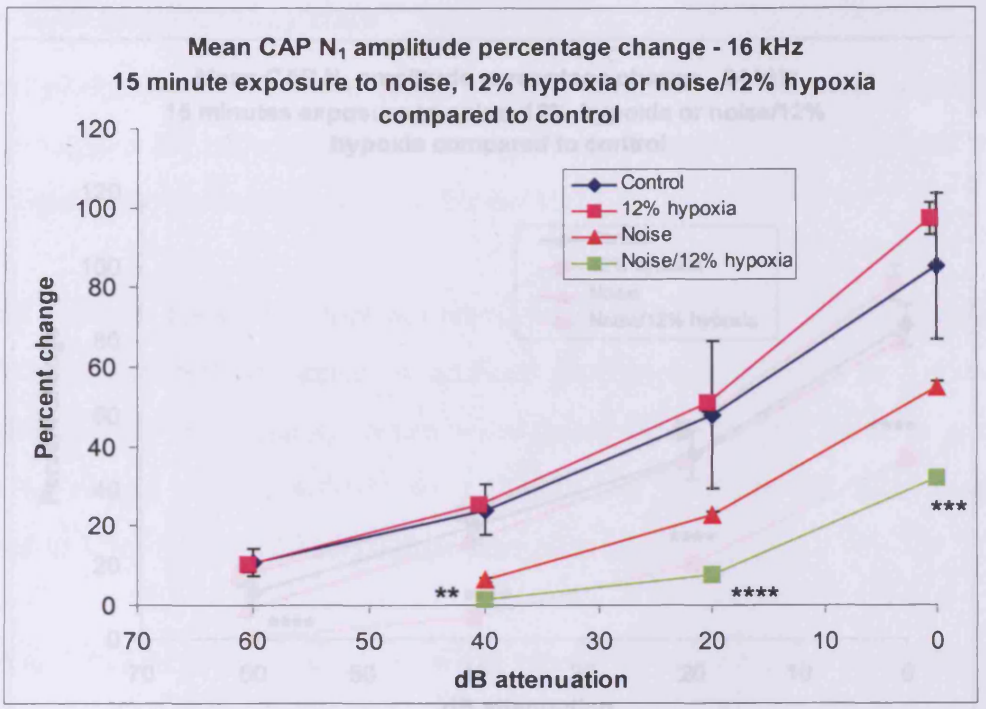


Figure 4.24. Mean CAP N_1 amplitude change (16 kHz) after 15 minutes exposure to 12% hypoxia, noise or NH compared to artificial perilymph control (n=4, 7, 10 or 7). $F_{3,22}$=35.72, $F_{3,22}$=27.02, $F_{3,22}$=62.21 and $F_{3,22}$=51.88, all P<0.0001 (0 to 60 dB attenuation). **p<0.01, ***p<0.001, ****p<0.00 t-test between noise and noise/12% hypoxia.

Figure 4.26 illustrates the less graded effects observed at 30 kHz, in line with those expected from the *within group* analysis for noise alone. Over 0 to 60 dB attenuation the mean control percentage values ranged from 82 to 11% for control, 75% to 9% for noise and 41 to 1% for NH. Statistical significance was achieved at all intensities for N vs NH at this frequency, with *post hoc* test p values of p<0.01, <0.001 and <0.05 (0, 20 and 40dB attenuation respectively). Amplitude changes after noise exposure ranged from 74.8± 7.9 to 9.3±1.7% compared to 41.1±7 to 1.6± 0.8% for NH. Control values were 81.9±8.1 to 10.8±1.8%.

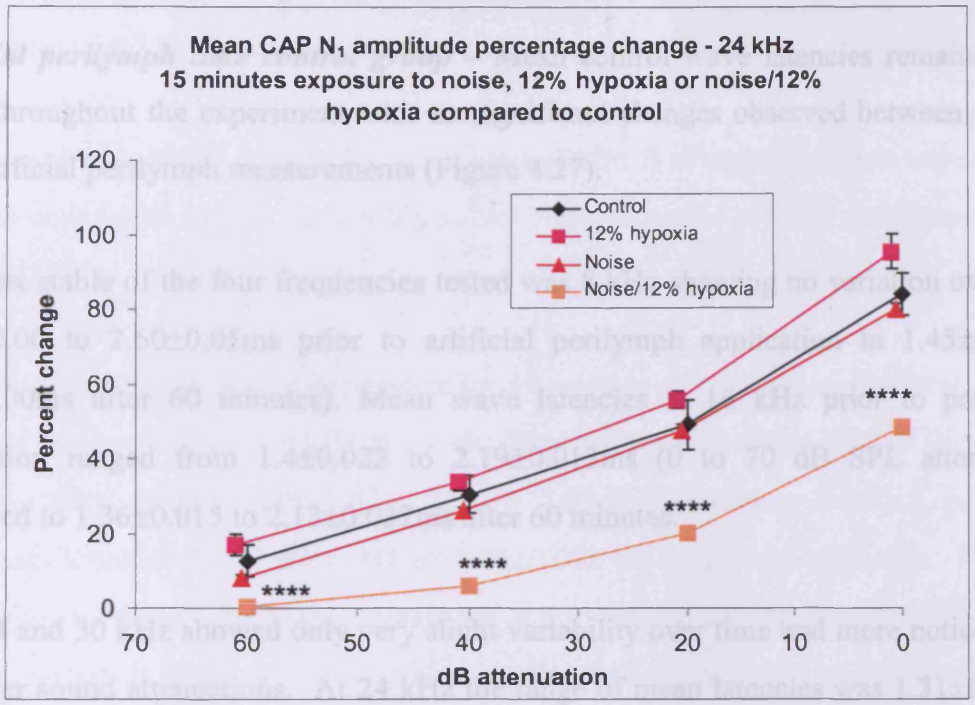


Figure 4.25. Mean CAP N_1 amplitude change (24 kHz) after 15 minutes exposure to 12% hypoxia, noise or NH compared to artificial perilymph control (n=4, 7, 10 or 7). $F_{3,22}$=17.06, $F_{3,22}$=13.63, $F_{3,22}$=20.1 and $F_{3,22}$=9.05, all P<0.0001 (0 to 60 dB attenuation). ****p<0.0001, t-test between noise and NH.

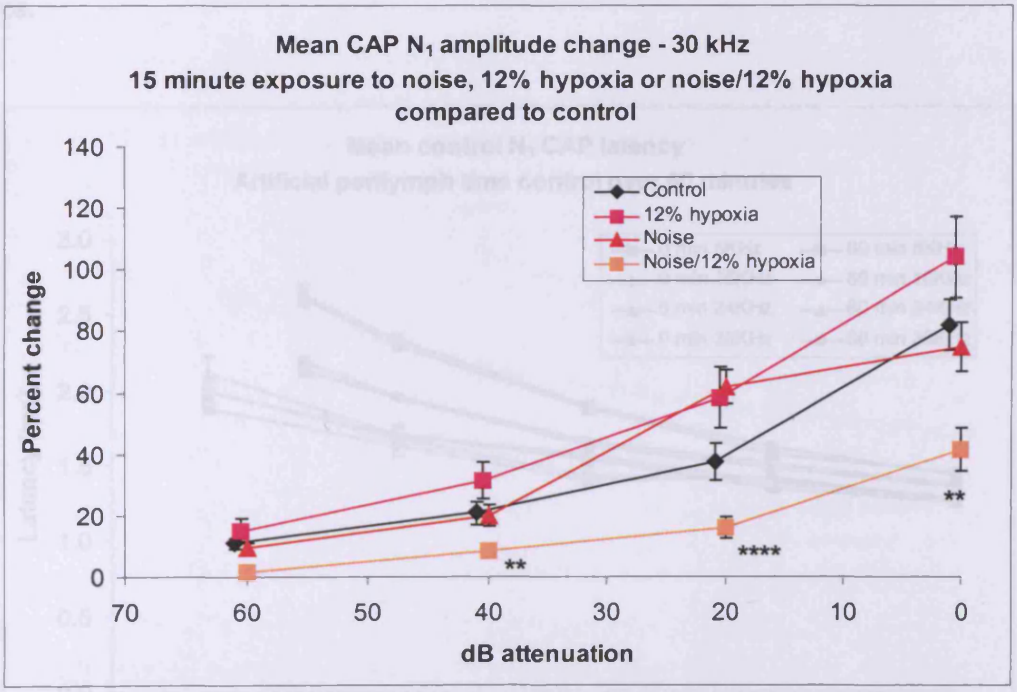


Figure 4.26. Mean CAP N_1 amplitude change (30 kHz) after 15 minutes exposure to H, N or NH compared to artificial perilymph control (n=4, 7, 10 or 7). $F_{3,21}$=10.15 P<0.0001, $F_{3,21}$=16.29 P<0.0001, $F_{3,20}$=7.26 P<0.01 and $F_{3,20}$=6.5 P<0.01 (0 to 60 dB attenuation). ****p<0.0001, **P<0.01, t-test between noise and NH.

4.11.3. CAP wave latency data

Artificial perilymph time control group – Mean control wave latencies remained very stable throughout the experiment with no significant changes observed between pre and post artificial perilymph measurements (Figure 4.27).

The most stable of the four frequencies tested was 8 kHz showing no variation over time (1.45±0.00 to 2.60±0.05ms prior to artificial perilymph application to 1.45±0.00 to 2.60±0.00ms after 60 minutes). Mean wave latencies at 16 kHz prior to perilymph application ranged from 1.4±0.022 to 2.19±0.013ms (0 to 70 dB SPL attenuation) compared to 1.36±0.015 to 2.13±0.032ms after 60 minutes.

Both 24 and 30 kHz showed only very slight variability over time and more noticeably at the lower sound attenuations. At 24 kHz the range of mean latencies was 1.31±0.032 to 2.04±0.033ms prior to artificial perilymph application, compared to 1.26±0.03 to 2.11±0.11ms after 60 minutes. Values for 30 kHz were 1.28±0.033 to 1.96±0.024ms prior to artificial perilymph application compared to 1.27±0.046 to 1.88±0.025ms after 60 minutes.

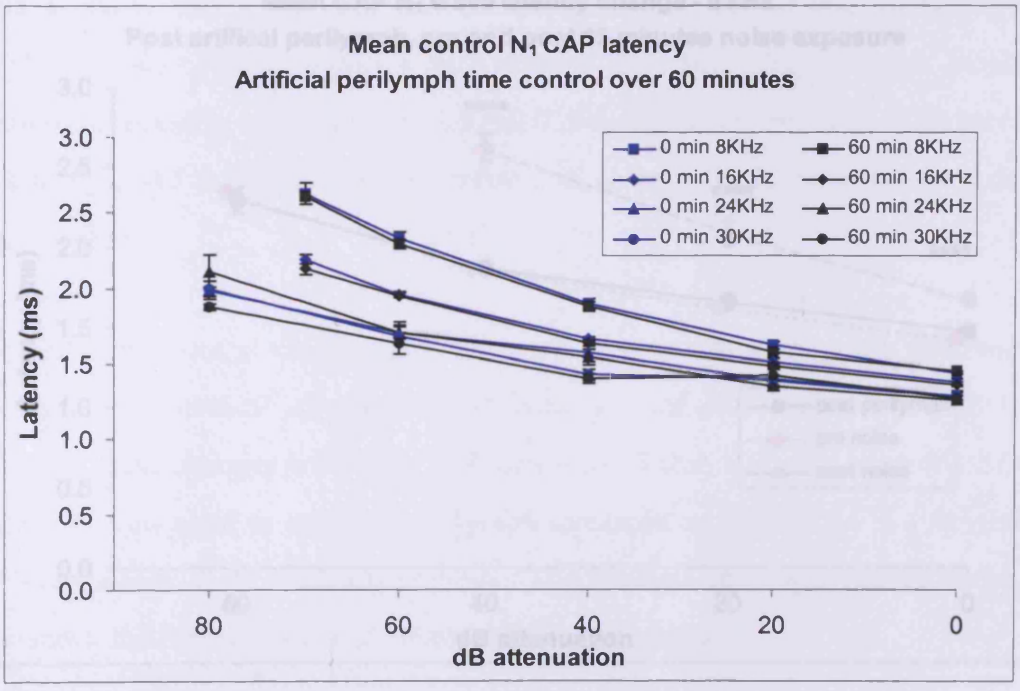


Figure 4.27. The very stable mean CAP N1 wave latency after application of artificial perilymph for 60 minutes (-15 minutes represents pre-artificial perilymph, n=7).

Exposure to 12% Hypoxia – No effects on wave latency were noted after exposure to 12% hypoxia for 15 minutes.

Exposure to 100dB SPL noise - The most marked effects of noise exposure was seen at 8 kHz. At both 8 and 16 kHz, no latency values were measured at 60dB attenuation due to threshold shifts.

Figure 4.28 shows that exposure to noise for 15 minutes produced statistically significant increases in wave latency at 8 kHz at 0 to 40 dB attenuation as shown by ANOVA. Mean wave latencies ranged from 1.47±0.021 to 2.29±0.084ms post artificial perilymph application increasing to 1.67±0.034 to 2.81±0.083 ms post noise exposure. Post hoc testing showed these to be highly significant at all levels of attenuation ($p<0.0001$). This effect on latency is likely to reflect an excitotoxic effect or metabolic overload on the CN. At 16 kHz, a highly significant increase of about 0.1 ms was seen at 40dB attenuation ($p<0.0001$). Mean wave latency ranged from 1.36±0.018 to 1.98±0.018ms post artificial perilymph application compared to 1.32±0.015 to 1.84±0.030ms post noise exposure (figure 4.29). Mean latencies for both 24 and 30 kHz were unaffected by noise exposure.

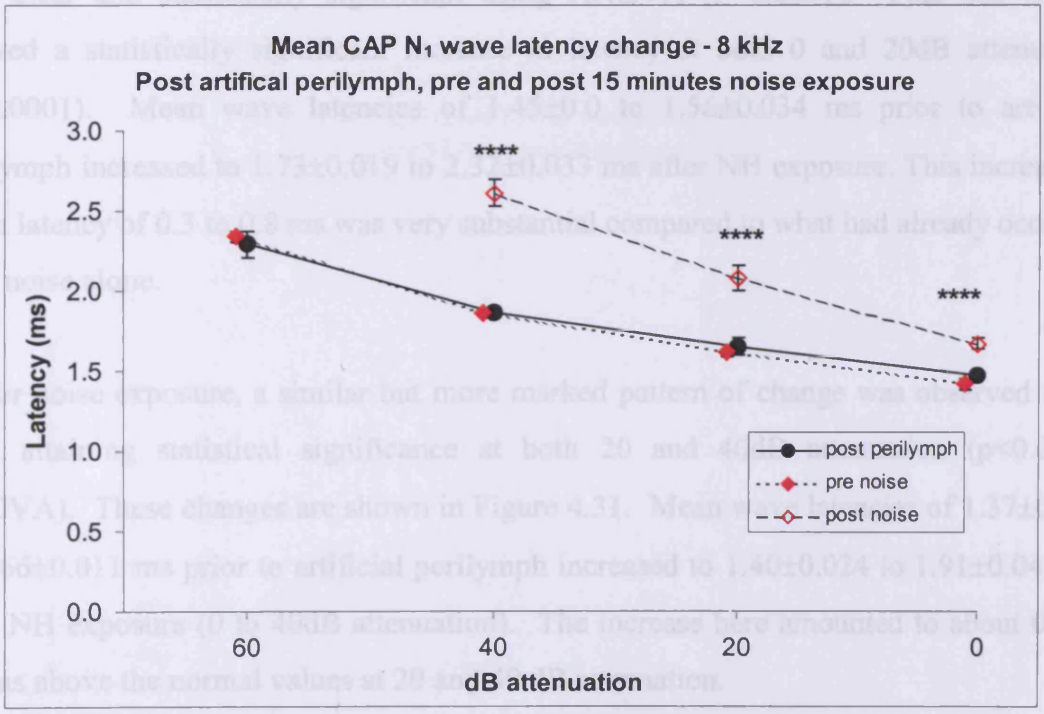


Figure 4.28. Mean CAP N_1 wave latency change (8 kHz) after 15 minutes exposure to noise (n=7). $F_{3,24}$=16.59, $F_{3,\ 24}$=20.82 and $F_{3,\ 24}$=84.87, all $p<0.0001$ (0 to 40dB attenuation). ****$P<0.0001$, t-test (artificial perilymph vs post noise). These increases were highly significant and indicative of an effect at the afferent synapse/CN

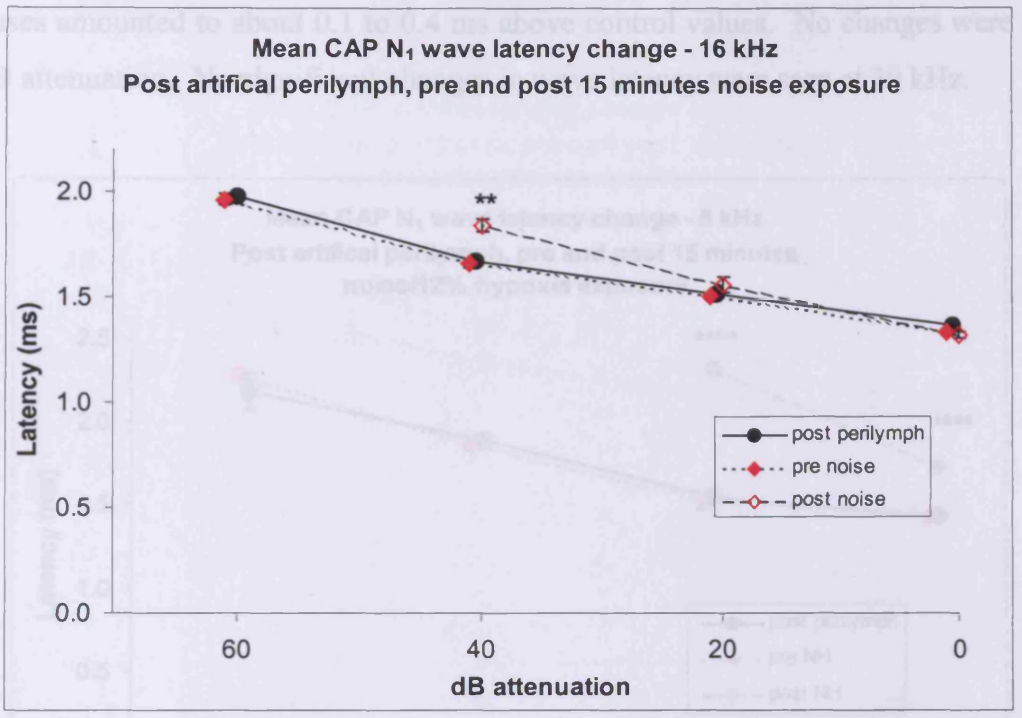


Figure 4.29. Mean CAP N_1 wave latency change (16 kHz) after 15 minutes exposure to noise (n=7). $F_{3,24}$=2.3 p=0.103, $F_{3,24}$=0.85 p=0.482 and $F_{3,23}$=13.0 p<0.0001 (0 to 40dB attenuation). **P<0.01, t-test (artificial perilymph vs post noise). The slight increase at 40 dB may reflect the mild excitotoxic/metabolic overload seen more extensively at 8 kHz.

Exposure to Noise/12% Hypoxia (NH) – Figure 4.30 shows that at 8 kHz, there was a very clear and statistically significant using ANOVA (P<0.0001). Post hoc testing showed a statistically significant increase in latency at both 0 and 20dB attenuation (p<0.0001). Mean wave latencies of 1.45±0.0 to 1.56±0.034 ms prior to artificial perilymph increased to 1.73±0.019 to 2.32±0.033 ms after NH exposure. This increase in mean latency of 0.3 to 0.8 ms was very substantial compared to what had already occurred with noise alone.

As for noise exposure, a similar but more marked pattern of change was observed at 16 kHz, attaining statistical significance at both 20 and 40dB attenuation (p<0.0001, ANOVA). These changes are shown in Figure 4.31. Mean wave latencies of 1.37±0.017 to 1.66±0.011 ms prior to artificial perilymph increased to 1.40±0.024 to 1.91±0.043 ms after NH exposure (0 to 40dB attenuation). The increase here amounted to about 0.1 to 0.2 ms above the normal values at 20 and 40 dB attenuation.

In contrast to noise exposure at 24 kHz, exposure to NH resulted in significant intensity dependent increases in mean latency (p<0.05 to p<0.01, ANOVA). In figure 4.32 these

increases amounted to about 0.1 to 0.4 ms above control values. No changes were noted at 0dB attenuation. No significant changes in wave latency were seen at 30 kHz.

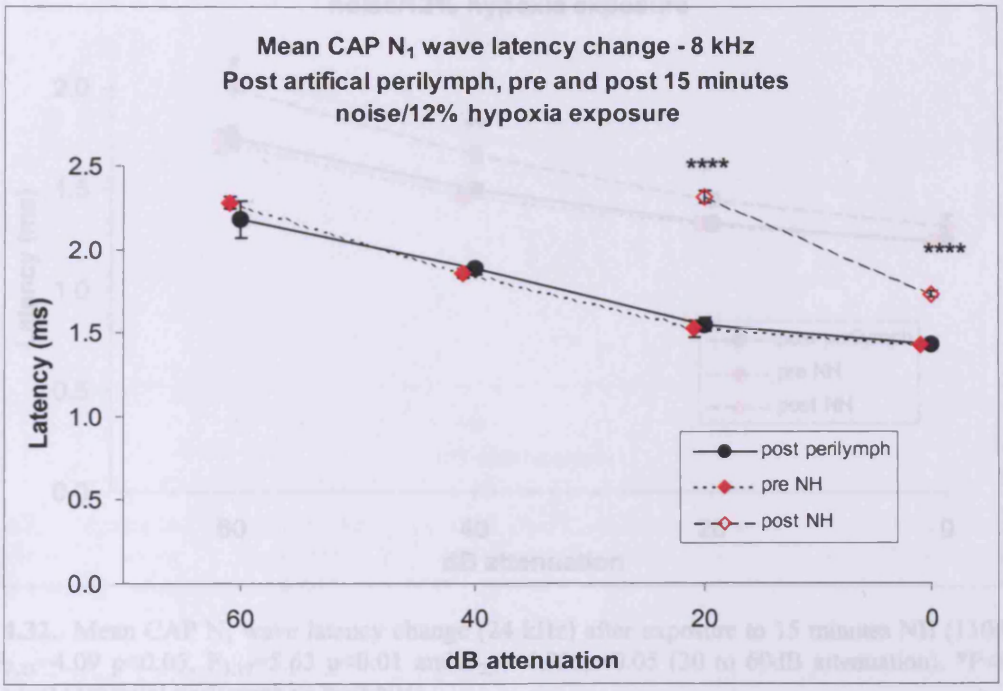


Figure 4.30. Mean CAP N_1 wave latency change (8 kHz) after 15 minutes exposure to NH (n=10). $F_{3, 31}$=106 and $F_{3, 30}$=102 both p<0.0001 (0 to 20dB attenuation). ***P<0.0001, t-test (artificial perilymph vs post NH).

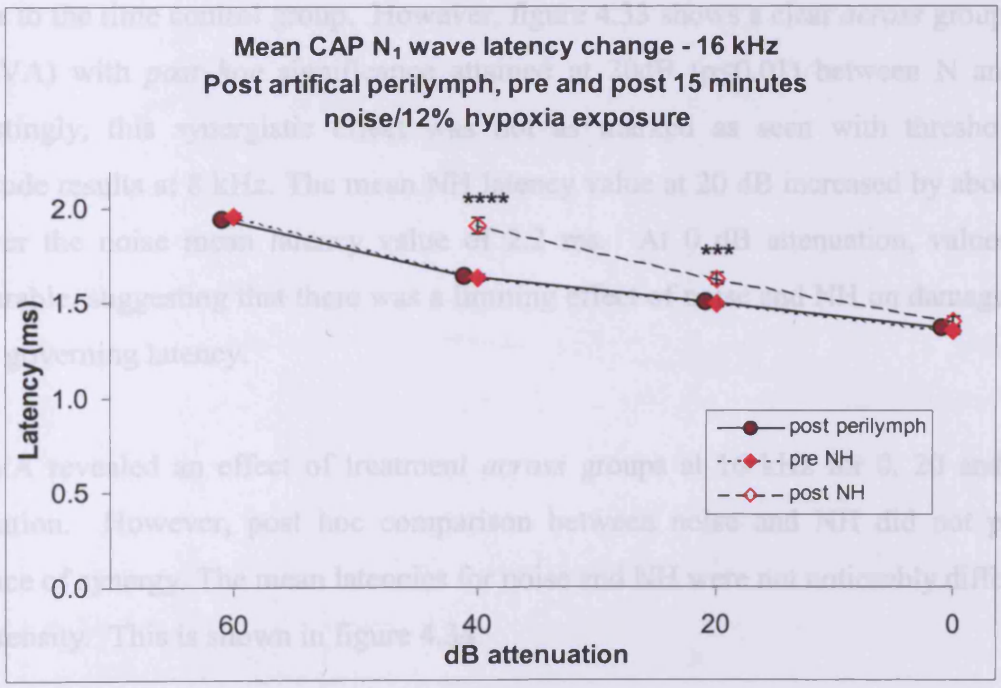


Figure 4.31. Mean CAP N_1 wave latency change (16 kHz) after exposure to 15 minutes NH (n=10). $F_{3,31}$=1.24 p=0.31, $F_{3, 30}$=14.28 and $F_{3,27}$=36.3, both p<0.0001 (0 to 40dB attenuation). ***P<0.001, **** P<0.0001 t-test (artificial perilymph vs post NH).

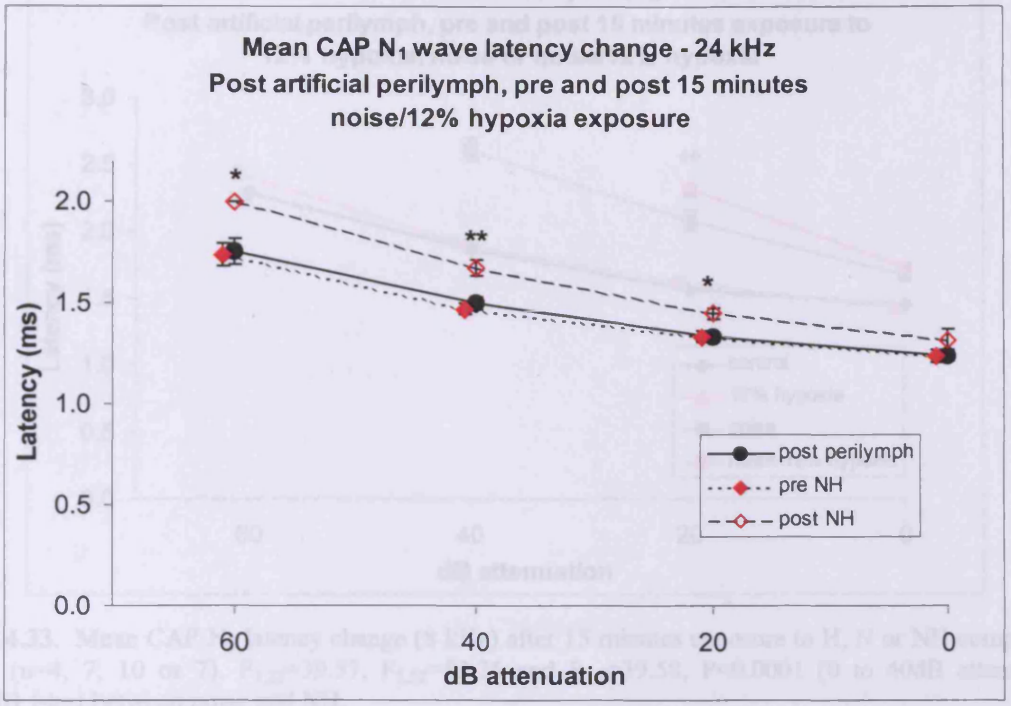


Figure 4.32. Mean CAP N_1 wave latency change (24 kHz) after exposure to 15 minutes NH (110dB SPL) n=10. $F_{3,21}$=4.09 p<0.05, $F_{3,19}$=5.63 p<0.01 and $F_{2,11}$=4.92 p<0.05 (20 to 60dB attenuation). *P<0.05, ** P<0.01 t-test (artificial perilymph vs post NH).

Across group analysis – At all frequencies, the response to 12% hypoxia was very similar to the time control group. However, figure 4.33 shows a clear *across* group effect (ANOVA) with *post hoc* significance attained at 20dB (p<0.01) between N and NH. Interestingly, this synergistic effect was not as marked as seen with threshold and amplitude results at 8 kHz. The mean NH latency value at 20 dB increased by about 0.15 ms over the noise mean latency value of 2.2 ms. At 0 dB attenuation, values were comparable, suggesting that there was a limiting effect of noise and NH on damage at the site(s) governing latency.

ANOVA revealed an effect of treatment *across* groups at 16 kHz for 0, 20 and 40dB attenuation. However, post hoc comparison between noise and NH did not provide evidence of synergy. The mean latencies for noise and NH were not noticeably different at any intensity. This is shown in figure 4.34.

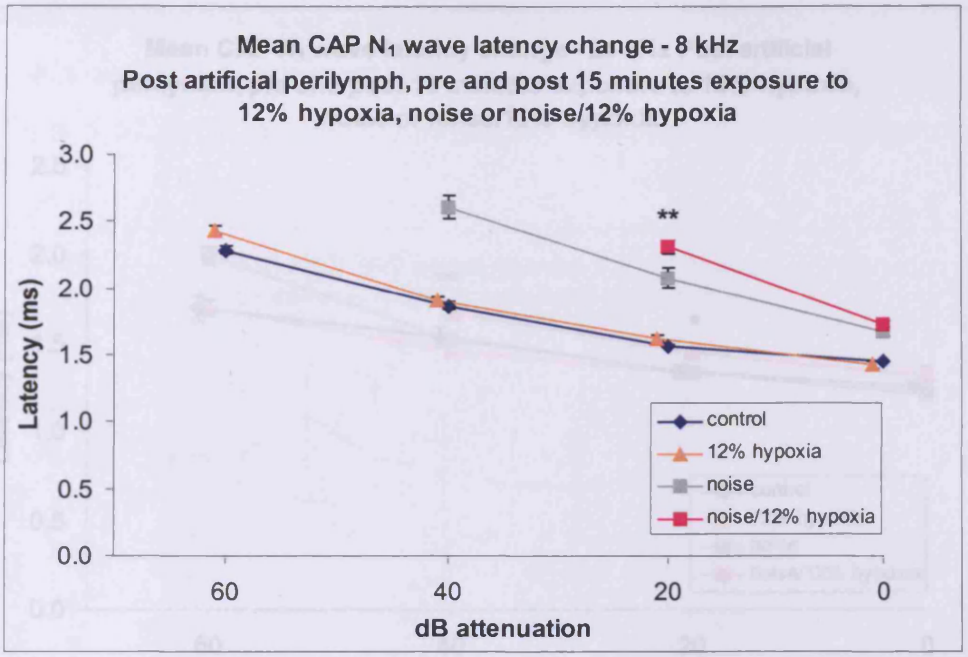


Figure 4.33. Mean CAP N_1 latency change (8 kHz) after 15 minutes exposure to H, N or NH compared to control (n=4, 7, 10 or 7). $F_{3,22}$=39.57, $F_{3,22}$=53.25 and $F_{2,9}$=39.58, P<0.0001 (0 to 40dB attenuation). **p<0.01 t-test between noise and NH.

At 24 kHz significance between groups was again evident but, as for 16 kHz, only at the lower sound stimuli. *Post hoc* comparison revealed differences between noise and NH at 20 and 40dB attenuation (p<0.05 and p<0.01 respectively) as shown in figure 4.35.

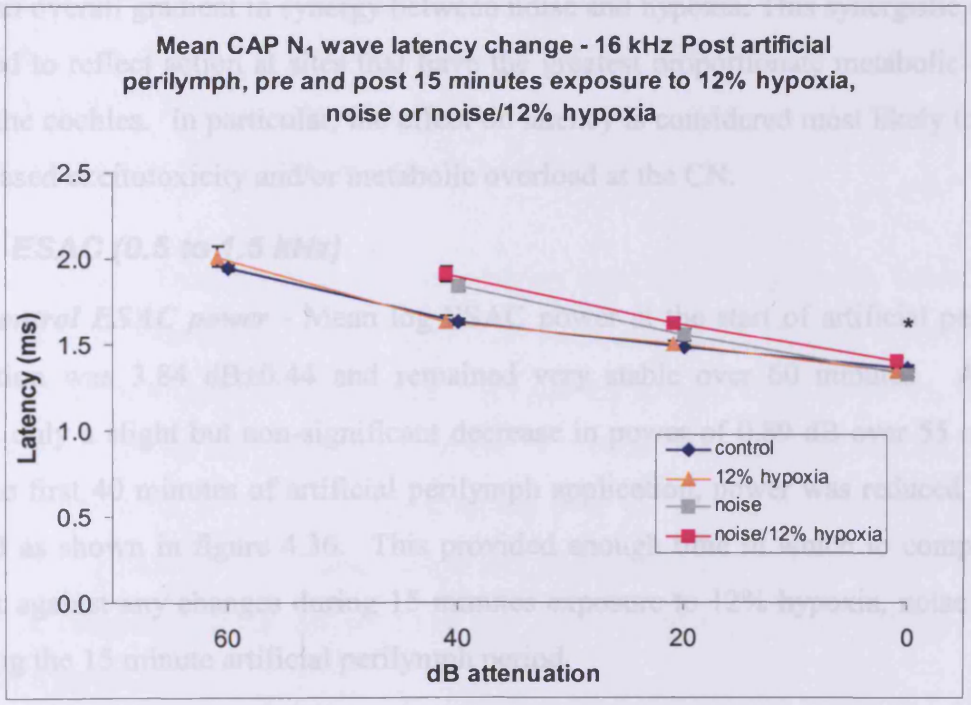


Figure 4.34. Mean CAP N_1 latency change (16 kHz) after 15 minutes exposure to H, N or NH compared to control (n=4, 7, 10 or 7). $F_{3,23}$=3.18 p<0.05, $F_{3,22}$=5.73 p<0.01 and $F_{3,17}$=17.97 P<0.0001 (0 to 40dB attenuation). *p<0.05 t-test between noise and NH.

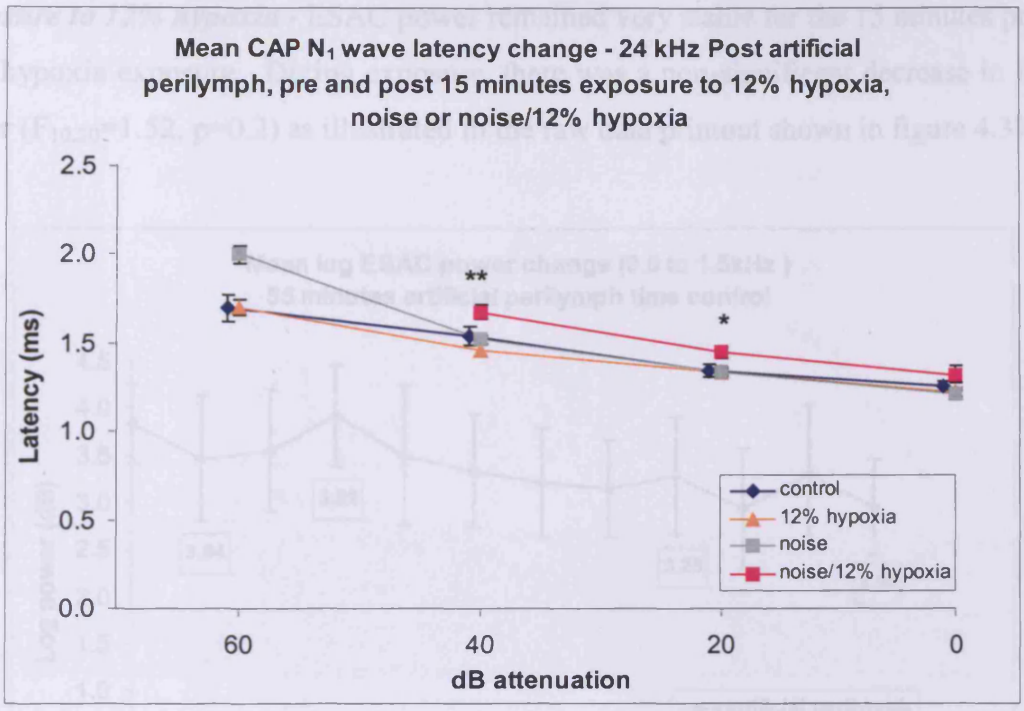


Figure 4.35. Mean CAP N_1 latency change (24 kHz) after 15 minutes exposure to H, N or NH compared to control (n=4, 7, 10 or 7). Using ANOVA, $F_{3,21}$=4.09, P<0.05, $F_{3,19}$=5.63, P<0.01 and $F2_{,11}$=4.92, P<0.05 for 20, 40 and 60dB attenuation respectively. *p<0.05, **p<0.01 t-test between noise and NH.

The increases in mean latency due to NH at this frequency were about 0.1 to 0.15 ms above those seen in control or noise treated animals. No evidence of across group differences in wave latency was noted at 30 kHz. Again, the effect of NH on latency shows an overall gradient in synergy between noise and hypoxia. This synergistic effect is proposed to reflect action at sites that have the greatest proportionate metabolic demand within the cochlea. In particular, the effect on latency is considered most likely to be due to increased excitotoxicity and/or metabolic overload at the CN.

4.11.4. ESAC (0.5 to 1.5 kHz)

Time control ESAC power - Mean log ESAC power at the start of artificial perilymph application was 3.84 dB±0.44 and remained very stable over 60 minutes. ANOVA showed only a slight but non-significant decrease in power of 0.89 dB over 55 minutes. Over the first 40 minutes of artificial perilymph application, power was reduced by only 0.56 dB as shown in figure 4.36. This provided enough time in which to compare and contrast against any changes during 15 minutes exposure to 12% hypoxia, noise or NH, including the 15 minute artificial perilymph period.

Exposure to 12% hypoxia - ESAC power remained very stable for the 15 minutes prior to 12% hypoxia exposure. During exposure, there was a non-significant decrease in ESAC power ($F_{10,20}$=1.52, p=0.2) as illustrated in the raw data printout shown in figure 4.37.

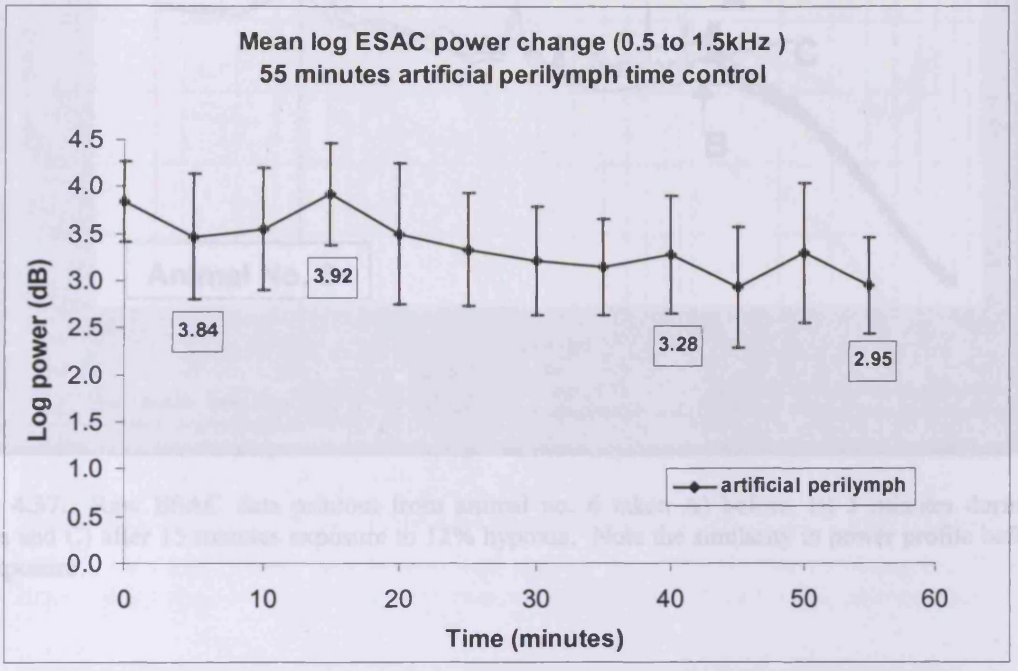


Figure 4.36. Mean log ESAC power change (0.5 to 1.5 kHz) over time after artificial perilymph application to the round window membrane (n=6). $F_{12, 62}$=0.79 P<0.661, ANOVA.

Exposure to noise or NH – During exposure to noise or NH it was possible to record changes in gross driven CN activity, referred to here as ensemble driven activity of CN activity (EDAC). Highly significant increases in EDAC power were observed in both groups over the exposure period as illustrated in figures 4.40 and 4.41.

Noise exposure – During noise exposure, highly significant increases in EDAC power where observed, followed by highly significant reductions in ESAC post exposure. This is shown in the raw data printout (figure 4.38) and the ESAC/EDAC power vs. time plot (figure 4.40). At the start of noise stimulus, a 4,100% increase in EDAC power was observed (equivalent to 17±0.65 dB) dropping over the 15 minute exposure period to 2,300% or 15±0.45 dB. Post noise exposure, an approximate 70% decrease in ESAC power was measured which did not show any evidence of recovery (p<0.001). This is shown in figure 4.41.

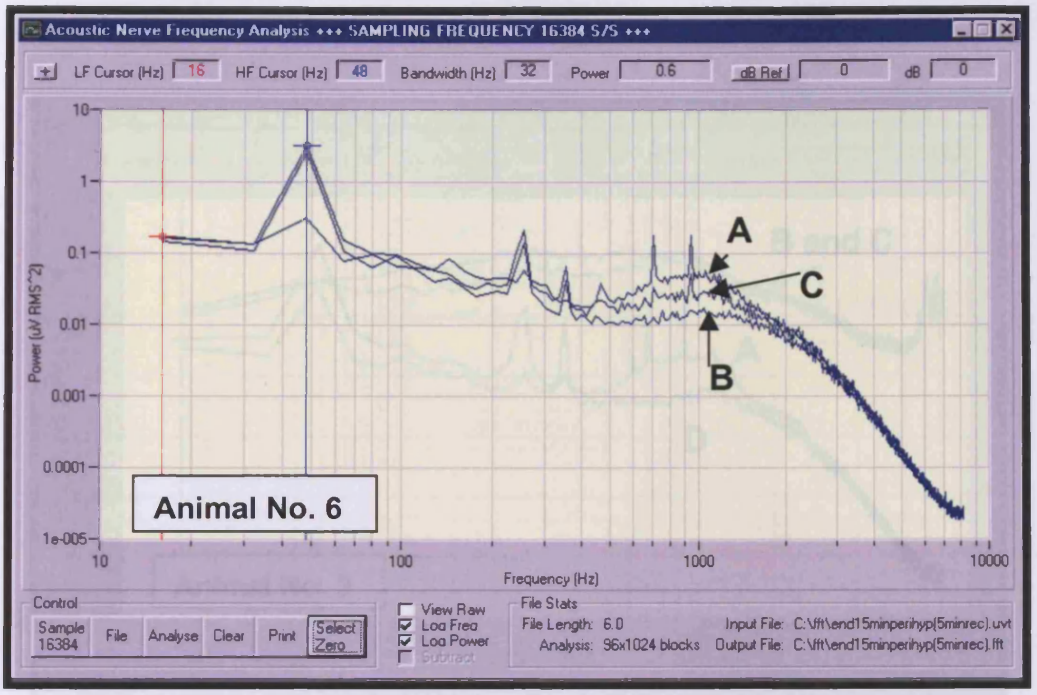


Figure 4.37. Raw ESAC data printout from animal no. 6 taken A) before, B) 5 minutes during 12% hypoxia and C) after 15 minutes exposure to 12% hypoxia. Note the similarity in power profile before and after exposure.

Noise/12% hypoxia (NH) – ANOVA revealed highly significant changes in EDAC power and post exposure ESAC power, which were very similar to those observed in the noise group. This is shown by the raw data printout in figure 4.39, and graphically in figures 4.40 and 4.41.

Across group comparison - ANOVA revealed significant differences between noise and NH compared to the controls, but there was no significant difference between the noise and NH groups. This is evidence that the driven and post exposure spontaneous nerve activity in these two groups were comparable (figure 4.40).

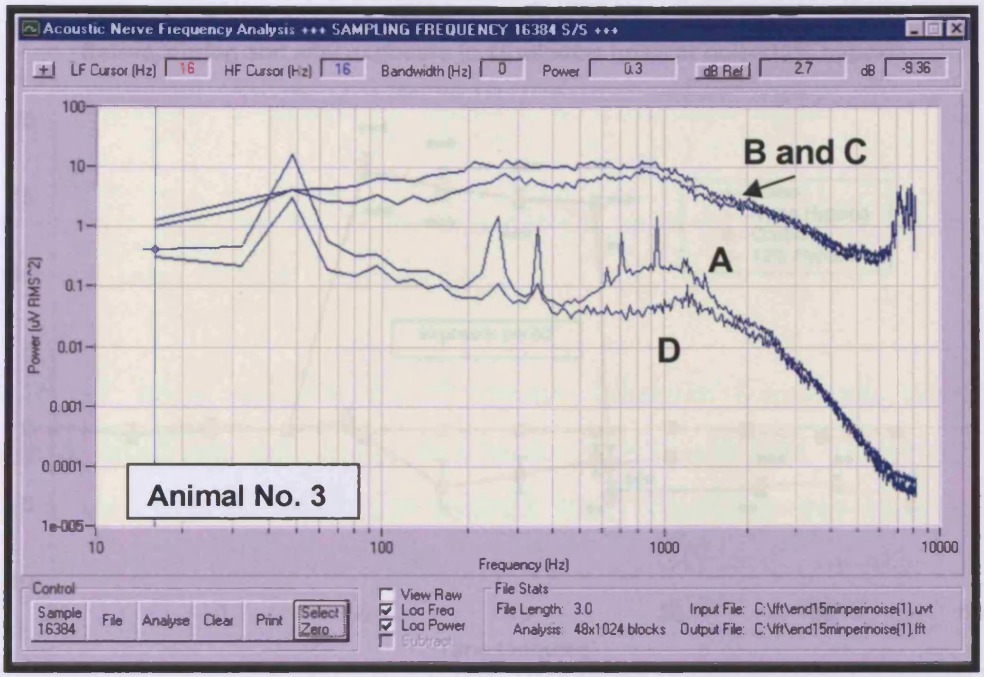


Figure 4.38. Raw ESAC/EDAC data from animal number A) before, B and C) during and D) after 15 minutes exposure to noise. Note the large reduction in power around 0.5 to 1.5 kHz after exposure.

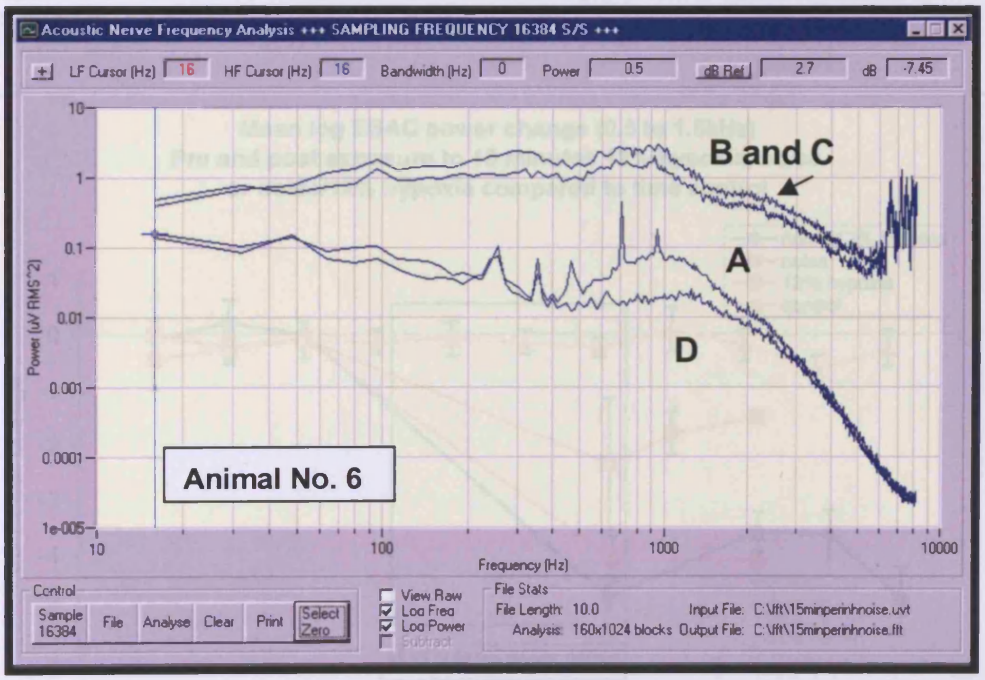


Figure 4.39. Raw ESAC/EDAC data A) before, B and C) during and D) after 15 minutes exposure to NH. As for noise, note the large reduction in power around 0.5 to 1.5 kHz after exposure.

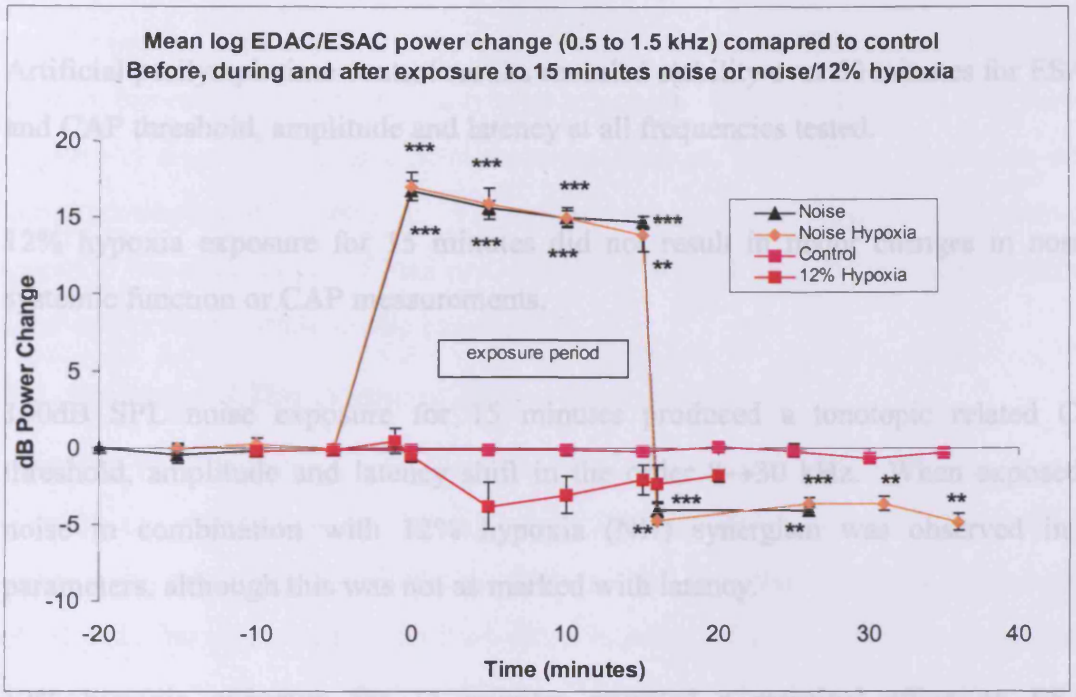


Figure 4.40. Mean log ESAC/EDAC power change compared to artificial perilymph control over time; pre, during and post exposure to 15 minutes 12% hypoxia, noise or NH (n=6, 4 and 10 respectively). Noise: $F_{10,50}$=376 p<0.0001, NH: $F_{11,58}$=198 p<0.0001. *** p<0.0001, ** p<0.001, t-test between control and either noise or NH exposure.

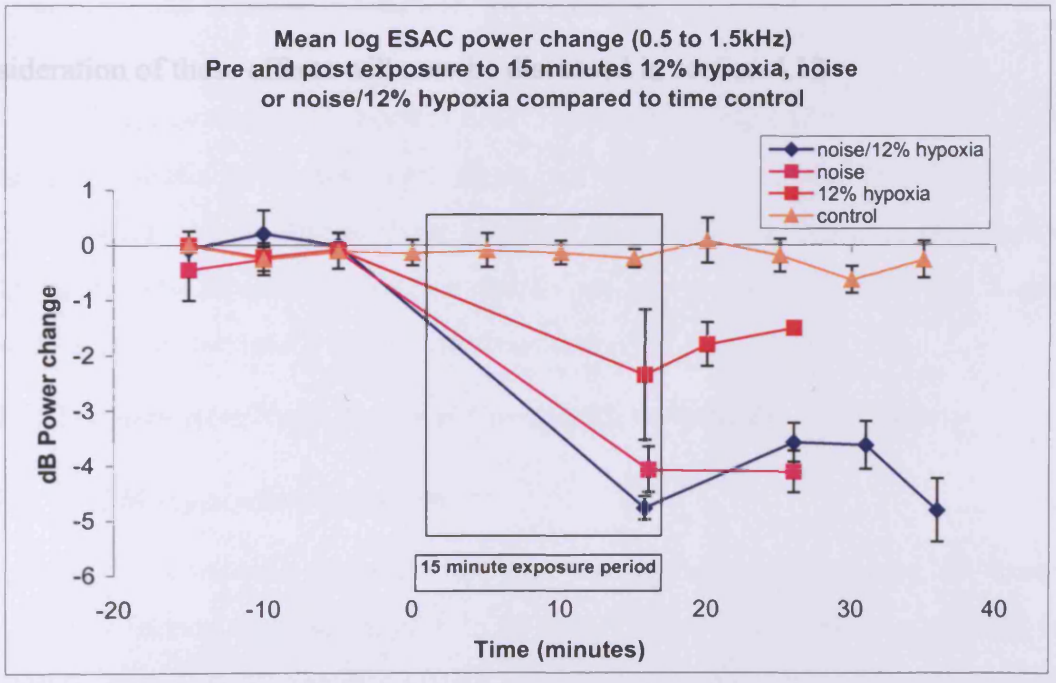


Figure 4.41. Mean log ESAC power change compared to artificial perilymph control over time; pre and post exposure to time control, 15 minutes 12% hypoxia, noise, or NH (n=6, 4 and 10 respectively).

In summary, the results from Chapter 4 showed the following:

- Artificial perilymph time control results revealed stability over 60 minutes for ESAC, and CAP threshold, amplitude and latency at all frequencies tested.

- 12% hypoxia exposure for 15 minutes did not result in major changes in normal systemic function or CAP measurements.

- 100dB SPL noise exposure for 15 minutes produced a tonotopic related CAP threshold, amplitude and latency shift in the order 8→30 kHz. When exposed to noise in combination with 12% hypoxia (NH) synergism was observed in all parameters, although this was not as marked with latency.

- 12% hypoxia exposure for 15 minutes revealed a marginal effect on ESAC. However, both noise and NH exposure produced a marked increase in EDAC power of approximately 4,000% compared to control, with a post exposure decrease of approximately 70%. No evidence of NH synergy was observed.

Consideration of these effects will now be discussed in section 4.12.

4.12. Discussion and conclusions

The results presented here provide new physiological clues as to the sites and mechanisms involved in the cochlea following acute noise exposure. Exposure to NH is considered to synergistically augment and accelerate the noise damage, especially at those sites which are more metabolically vulnerable. These ideas are discussed below, and in particular compared to the acute model of Puel *et al* (1988, 1998) with regards to the contribution of physical and excitotoxic/metabolic overload.

4.12.1. Stability of Time Control data – CAP and ESAC

The artificial perilymph time control results for CAP threshold, amplitude and latency at all frequencies were very stable throughout the recording period. An important observation, however, was an immediate drop in CAP amplitude of about 40% routinely observed just after placement of the fluid-filled IntraEar E-Cath®. This was considered to be primarily due to changes in the additional electrical capacitance and resistance path introduced by the artificial perilymph reservoir rather than any real physiological effect. This was supported by the absence of changes in both CAP threshold and latency, as both would have been expected to be affected if there had have been a real physiological rather than just a physical effect of the catheter placement.

Artificial perilymph results for control ESAC were also stable and again showed a similar drop in peak amplitude of about 40%, again just after catheter placement. In appearance, the results were very similar to those obtained by Cazals and Huang (1996) showing a trough in activity around 0.4 kHz, a 'hump' in power around 1 kHz and a gradual decrease in power over the 8 kHz spectral window.

4.12.2. Treatment effects on CAP threshold, amplitude and latency

4.12.2.1. 12% Hypoxia exposure

Exposure to 12% hypoxia alone did not have any significant effects on CAP threshold, amplitude or latency over the range 8 to 30 kHz. These results are in agreement to the findings by Nuttall and Lawrence (1980) as shown in figure 4.8, and Gafni and Sohmer (1976) who showed no effect on EP above 10-12% oxygen. In addition, it was established in Chapter 3 that no marked effects in basal functions were apparent at this level of hypoxia. This meant that there was no likelihood of an effect due to a more general systemic, cardiovascular or pulmonary functional deficit on cochlear evoked

responses (Evans 1972, Mulheran 1990). The clear absence of an effect on CAP threshold suggests that the metabolically more sensitive processes involved in transduction downstream from the EP were also unaffected (Russell and Cowley 1983, Brown *et al* 1983, Ryan 1988). However, Nuttall and Lawrence (1980) and Gafni and Sohmer (1976) did not provide any evidence of the effects of 12% hypoxia on *evoked* cochlear activity.

The results presented here, show for the first time that acute cochlear performance in the guinea pig remains intact in the presence of 12% hypoxia as measured by CAP. This provides further justification for the choice of 12% hypoxia as a synergistic agent with noise. During co-presentation with noise, any synergy was less likely to be due to physical trauma and was more likely to be due to enhancement of excitotoxic/metabolic damage.

4.12.2.2. Noise exposure

In line with the BAER data generated in Chapter 3, the acute 100dB SPL noise exposure produced a tonotopically related threshold shift with marked elevations of about 25dB at 8 and 16 kHz, about 12 dB at 24 kHz, and a less marked 9 dB shift at 30 kHz. The spread of significant threshold elevation up to 24 kHz and beyond the bandpass characteristics of the noise stimulus is explainable in terms of the half octave frequency shift in maximal damage. This is shown schematically in figure 4.6 and has been described by various workers (Henderson *et al* 1983, Salvi *et al* 1979 and Puel *et al*, 1988).

In this case, the half octave spread of traumatic stimuli is compounded by noise at different levels within the presentation bandwidth. Consequently, the half octave shift seen more clearly with single tone noise is 'smeared out' in a more graded manner. The results in this chapter show that brief, moderate intensity, broadband noise in the albino guinea pig, can be used to produce substantial acute trauma. A further strength of this model is that a range of threshold deficits can be produced, whilst minimising involvement of marked physical damage in the cochlea (Puel *et al* 1988, 1998).

Effects of noise on amplitude at 8 kHz - The effects of noise on amplitude, when taken together with the effects of threshold over 8 to 24 kHz, showed a graded range of effects. These reflect the differing degree of noise insult due its bandpass characteristics illustrated in figure 4.1. The greatest effect was seen at 8 kHz, which fell in the part of

the noise stimulus spectrum with the greatest level of intensity over 5-10 kHz. At the lower intensities of 20 and 40dB attenuation, there was a very marked reduction in amplitude, which returned to control values at 0dB attenuation. This can be explained in terms of damage to the two components of OHC function in the normal cochlea (Liberman and Kiang, 1984, Kossl and Russell, 1992).

The lower stimulus intensities in the normal cochlea are augmented by the *active amplification* process (Neely and Kim 1983, 1986). In this study, at 8 kHz, this process has been clearly damaged (Kossl and Russell, 1992). At higher stimulus intensities, the intact organ of Corti then also acts to *actively attenuate* BM movement. This provides a limit to CN output and in turn affords some degree of metabolic protection (Liberman and Kiang 1984c, Ryan 1988).

If, along with the *active amplification* stage this *active attenuating* process is damaged, then high intensity stimuli can result in an amplitude response equal to or greater to that seen in the normal cochlea. This would be caused by the unmasking of the *passive* resonance of the BM, which has a steeper growth rate. Therefore, the appearance of the CAP amplitude at 8 kHz following noise exposure is proposed, in part, to be due to damage to both the *active amplifying* and *attenuating* processes.

Effects of noise on amplitude at 16 - 30 kHz - In contrast, the effects seen at 16 and 24 kHz suggest that the *active amplification* process had been affected, but the active attenuating process still remained intact. This is supported by the slope of the input-output function generally remaining parallel to that seen with the controls. This can be taken to indicate some degree of physical or physiological damage that was reflected in a moderate (12-25 dB) reduction in sensitivity. At 30 kHz, there was evidence of non-significant elevation in threshold reflecting lesser effects on the more sensitive part of the *active* amplification process. However, at supra threshold intensities there was no evidence of an effect on the ability of this region of the cochlea to generate a CAP response.

On their own, these effects on amplitude have been explained by physical damage to, and/or physiological deficit in the cochlear amplifier (Puel *et al* 1988, 1998). Although

not ruling out an excitotoxic/metabolic overload component, the results here would at first viewing be primarily explainable by damage to the OHC or cochlear amplifier stage.

Effects of noise on latency - Marked significant increases in latency were observed at 0 to 40 dB attenuation for 8 kHz, but at 16 kHz this increase was less marked and seen at 40 dB attenuation only. There was no evidence of increases in latency at either 24 or 30 kHz again reflecting the band pass nature of the noise stimulus. Whilst this latency increase with noise alone may be of mixed origin, it is considered here as tentative initial evidence of moderate acute excitotoxicity/metabolic overload.

Direct comparison of these results with the previous literature is made difficult because of differences in model, species and stimulus intensity/duration. Probably the most relevant comparison of the results in this chapter are with those of Puel *et al* (1988, 1998) who also studied acute levels of noise exposure (95 dB SPL) for 15 minutes. However, full comparison is limited as Puel and co-workers used a single 6 kHz tone stimulus with pigmented guinea pigs, whilst albino guinea pigs and a broadband noise stimulus were used in this thesis. Notwithstanding these differences, the peak elevations of about 20-25 dB at 8.5 kHz reported by Puel *et al* (1988, 1998) were comparable with the peak elevations of 25 dB at 8 and 16 kHz reported in this chapter. This suggests that, in spite of the differences in noise spectrum, the ability of the insult to produce acute elevation in threshold is comparable. In addition to this, Puel *et al* also found at the frequency most affected (8.5 kHz) the amplitude responses to lower intensity stimuli of between 40 to 75 dB SPL were significantly depressed, but at higher stimulus intensities, values were comparable to the controls. This is in line with the results at 8 – 24 kHz from this study, and is in agreement with the proposal by Puel *et al* that acute noise effects the cochlear *active* process.

The nature of the increase in latency reported here primarily at 8 kHz is in partial contrast with other workers (Salvi *et al* 1979b, Puel *et al* 1988). For example, Salvi *et al* (1979) reported no suprathreshold increases in single unit latency in chinchillas. Here, threshold shifts of between 20 - 40 dB were recorded over 2 - 15 hours following 5 days of low frequency noise exposure. Interestingly though, Puel *et al* (1988) reported increases in latency of 0.1 to 0.3 ms which were comparable to those reported in this thesis, albeit at 6 kHz, over a similar stimulus intensity range. The authors proposed that this increase was

due to physical and or physiological damage to the more basal higher frequency region of the cochlea where there is maximal acute threshold elevation. This is instead of excitotoxic damage or metabolic overload at the CN.

4.12.2.3. Noise/12% Hypoxia (NH) exposure

Noise/12% hypoxia (NH) exposure resulted in highly significant shifts in threshold, amplitude and latency. These results provided strong evidence for synergism between noise and hypoxia exhibiting a tonotopic gradation.

The synergistic effects of NH - Marked and significant threshold elevation above those seen with noise alone were seen across all frequencies. The mean increases above noise amounted to 13, 9, 16 and 6 dB for 8, 16, 24 and 30 kHz respectively. Notably, at 30 kHz the synergy had the effect of turning a non-significant threshold increase into a highly significant one. These changes were coupled with severe depression in amplitude at all stimulus intensities. With latency, highly significant intensity and tonotopically dependent increases were seen that extended up to 24 kHz. The most likely sites for this synergy are considered most likely to involve those sites with greatest metabolic vulnerability.

Effects of NH at 8 kHz - Threshold increases coupled with a very marked depression in amplitude were clearly evident. In terms of an effect on the active cochlear processes, ie. at the level of the OHCs, this could be interpreted as a greater demand being placed at the 8 kHz locus due to a reduction in oxygen supply. Following an 85 dB SPL noise insult, an increase in OHC glucose demand of about 10% has been shown (Ryan 1988) so it is feasible that 12% hypoxia could further contribute to this deficit in increased energy demand being met by the OHCs. It would not, however, be expected to affect the EP, which is the immediate source of the electromotive force for the active process as described above (Nuttall and Lawrence, 1980, Gafni and Sohmer, 1976).

The effect seen at 8 kHz was principally proposed to be due a physical/physiological effect on the active process, leading to lower amplitudes up to 20 dB attenuation. Above this the effect of noise was to *unmask* the passive growth function of the BM. If this were correct, then as a *passive* process it would not be expected to be directly affected by hypoxia. This does not rule out a hypoxic effect at the OHC completely, but the synergistic effect on threshold and amplitude is more likely to occur at the metabolically

more vulnerable IHCs, afferent CN and the glial cells (Russell and Cowley 1983, Brown *et al* 1983, Ryan 1988, Furness and Lehre 1997). The modest but synergistic increase in latency would also provide intersupporting evidence here for involvement of one or more of these vulnerable sites.

Effects of NH at 16 - 24 kHz - Clear synergistic elevation of threshold and suppression of amplitude were seen at 16, 24 and 30 kHz, with the most marked effect seen at 24 kHz. This could be taken as evidence of an augmented hypoxic effect on the active process, particularly on the amplifier stage operating at lower stimulus intensities which explains the graded elevations in thresholds and depression in amplitude. However, acute performance deficit at the IHC afferent CN and glial cells is proposed here to contribute to the observed effect.

Synergistic evidence from CAP latency results between noise and NH was curiously less marked and observed at 8 and 24 kHz, but not 16 kHz. However, following NH exposure there was a significant increase in latency above that seen in control animals, and at intensities where there had been no significant increase with noise alone, especially at 24 kHz. This was also the frequency at which there was there was maximal synergistic elevation in threshold. At 30 kHz, no increases in latency were seen with NH.

Summarising the above, the effect of NH on CAP threshold, amplitude and latency could include a synergistic effect on the OHCs and could explain the increases in threshold and decreases in amplitude seen at 16-30 kHz. Taken with the partly synergistic part NH alone increases in latency, this points to a greater probability of involvement of the metabolically more vulnerable processes.

4.12.3. Effects of noise and NH on ESAC and EDAC

Control ESAC recordings were very stable with 12% hypoxia having a marginal but non significant effect on ESAC power. In contrast with the changes observed in CAP measurements, there was no evidence of synergy between noise and 12% hypoxia. Both noise and NH regimens produced very large, but effectively identical changes in EDAC power over the exposure period of approximately 4,000%. This suggests that, under these conditions, output from the CN was near saturation and was not further affected by NH. It should be noted that the EDAC stimuli were continuous in contrast to the discrete

evoked stimuli used for CAP responses. Elucidation of these differences would, however, be provided by single unit CN recordings.

What is also of considerable broader scientific interest is that these results provide a non invasive way of indirectly assessing metabolic demand in the nerve. The 40-fold increase in power represents the electrical power due to ionic movement in the CN. How this directly relates to ATP is not known, but it is likely to represent a massive increase in demand. This demand is from along the whole peripheral end of the neurone and can stretch some tens of microns from the cell body (Santi, 1988). This is of considerable relevance as it shows what metabolic stresses the CN is placed under during prolonged high levels of stimulation. This provides further evidence about the degree of metabolic overload experienced by the CN following noise exposure.

Interestingly, post exposure ESAC results suggest that in both the noise and NH groups, spontaneous activity was equally reduced by approximately 70%. Whilst there was no evidence of synergy, this does suggest in both cases that there was a deficit due to either excitotoxicity and/or metabolic overload. Physiologically, the depression in ESAC power suggests that the afferent nerve fibres are partially 'burnt out' and their ability to carry normal neural signal has been significantly compromised. This does not detract from the robust evidence of synergistic interaction provided by the evoked CAP response. It does, however, provide inter supporting evidence along with the CAP results that the CN is a separate site for damage following noise exposure.

It is of direct relevance to this study that many humans, either occupationally or recreationally, are routinely and repeatedly exposed to levels of noise (or music) equivalent to those employed in this study. It is this exposure that leads in considerable part to the search for pharmacological therapy to prevent or ameliorate hearing deficit.

4.12.4. Provisional model of damage to the cochlea following NH

Physical/physiological and excitotoxic/metabolic overload components - On their own, these results provide evidence of an excitotoxic/metabolic overload component to the observed acute cochlear deficit. The proposal for involvement of an excitotoxic component due to noise is not new and much of the output from Puel and co-workers supports this. What is novel though, is the possibility that their may be an

excitotoxic/metabolic component following the acute duration and stimulus levels employed here. Stronger supporting evidence for this proposed excitotoxic/metabolic involvement with noise alone is presented in Chapter 5.

Involvement of physical/ physiological deficit in IHCs and OHCs - Previous acute studies have tended to emphasise the physical damage that immediately follows noise exposure (Liberman *et al*, 1982, Morest 1982, Henderson and Hamernick 1985, Puel *et al,* 1988, 1998 and Sullivan and Conolly, 1988). This physical damage was characterised by gross ultrastructural changes in the OHCs and supporting structures. The involvement of this component is not in question though the relative contribution it makes dependent on duration and intensity in acute exposures is not well characterised.

In addition to these physical changes there is also the possibility that acute physiological changes in both IHCs and OHCs may occur, as described by Cody and Russell (1985, 1988). This involves the loss of asymmetry in the IHC apical channel transfer function with decreased receptor potential. In the OHCs, depolarisation would occur due to excessive K^+ currents. Both these changes would be expected to lead to an acute increase in threshold and decreases in amplitude.

Involvement of the efferent nerve system - Within any acute noise model, there is the possible and likely contribution made by the efferent nerve system acting to limit damage. However, these limiting actions would potentially compound those effects seen with noise and NH. The medial efferent pathway, mediated by ACh and possibly GABA, would act to uncouple the OHCs (Pujol, 1993, 1994) whilst the lateral efferent pathway, most probably via dopamine, could act to decrease sensitivity and the driven rate of the afferent CN fibres (Ruel *et al,* 2001). This important recent work by Ruel *et al* showed that following intracochlear perfusion of 1mM dopamine, CAP thresholds amplitudes could be increased by about 10 dB or so and latency increased by 0.3–0.4 ms. A failing of these experiments was that they did not demonstrate the effect of *in vivo* concentrations of dopamine on CN activity. Moreover, the synergistic effects seen with NH would not be wholly explainable just because of increased activity in the efferent system as discussed below. The putative protectant effect of the efferent nerve fibres also appears to be limited in longer term exposures, which is borne out by the repeated findings of physical

damage and excitotoxic damage seen in other acute and longer term models of noise exposure.

Involvement of metabolically vulnerable sites - The results obtained with noise alone at 8 kHz and at all frequencies with NH, support the idea of involvement of sites that are particularly susceptible to metabolic overload. These sites would especially include the IHCs and their associated glial cells, and the IHC afferent fibres (Russell and Cowley 1983, Brown *et al* 1983, Ryan 1988, Furness and Lehre, 1997). The work reviewed by Ryan (1988) showed a very clear hierarchy in terms of metabolic demand during 85dB SPL noise stimulation with relative increases in glucose utilisation of 220% for the CN, 160% for the IHCs, 40% for the stria vascularis and lastly, 20% for OHCs.

Glial cells - The glial cells are less vulnerable to disruption in glucose and oxygen supply, showing only about a 25% increase in glucose demand following 85 dB SPL white noise (see figure 4.5, supporting cells). They are, however, uniquely vulnerable to Na^+ overload and would be expected to respond by *outward* Na^+ transport due to disruption of GLAST activity (Hyodo *et al* 1987, Stys *et al* 1992, Kiedrowski *et al* 1994 a-b). This is partly supported by figure 4.42, which shows an increase in cochlear perilymph glutamate levels following 15 minutes of marked hypoxia but without noise. This led to an increase of about 40 dB in CAP thresholds (Hyodo *et al*, 2001).

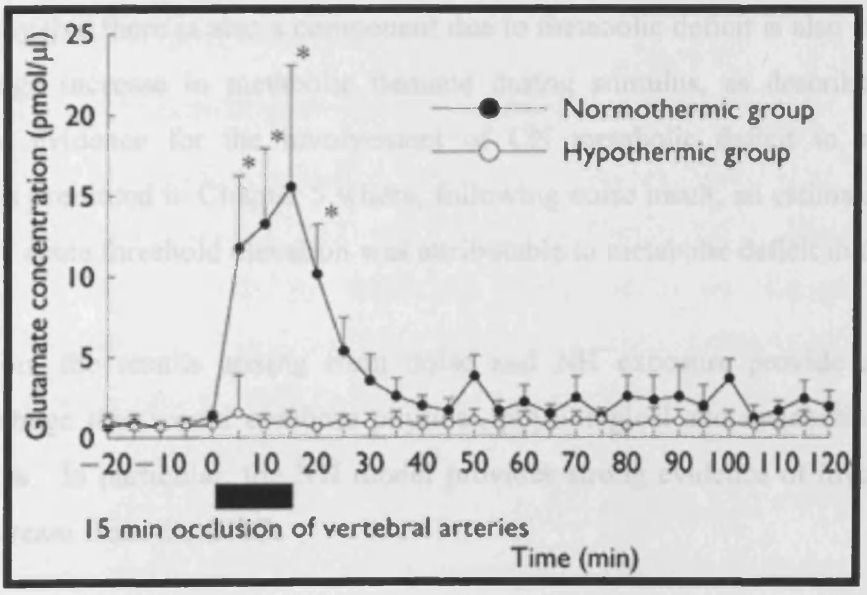


Figure 4.42. The increase, and accumulation in perilymphatic glutamate concentration during a period of total ischaemia. Taken from Hyodo *et al*, 2001.

If GLAST function were disrupted by NH insult, this would be very similar to increased noise stimulus, because the glutamate concentration in the synaptic cleft would be both increased and prolonged. Further evidence highlighting the importance of GLAST in the cochlea has been demonstrated by Hakuba *et al* (2000) who reported that mice lacking this transporter showed an increased glutamate concentration in the perilymph following a 30 minute exposure to a 105 dB SPL, 4 kHz pure tone. In this case the glial cells were a secondary target, as their role in buffering glutamate was likely to be compromised principally by excessive Na^+ uptake. This would then lead to prolonged and increased levels of glutamate in the afferent synaptic cleft.

IHCs - Apart from that already described above, NH damage at the IHC site is also likely to involve a further reduction in K^+ and Ca^{2+} ion handling and removal. This would explain the prolonged recovery of the IHC resting potential following acute hypoxia (Russell and Cowley, 1983). The resultant outcome from this would be to potentially add to any reductions in the dc receptor potential due to noise alone (Cody and Russell, 1983).

Afferent CN fibres - The involvement of the afferent CN fibres in both acute noise and NH models has been provided by Puel *et al* (1988, 1998), Chen *et al* (1999, 2001) and Selvadurai *et al* (2001), clearly showing that damage to the nerve could be ameliorated using both non-NMDA and NMDA glutamate antagonists. The nature of the damage at the nerve has previously been considered to be primarily excitotoxic in nature. However, the possibility that there is also a component due to metabolic deficit is also suggested by the very large increase in metabolic demand during stimulus, as described by Ryan (1988). The evidence for the involvement of CN metabolic deficit to support this hypothesis is presented in Chapter 5 where, following noise insult, an estimated 10 dB of the observed acute threshold elevation was attributable to metabolic deficit in the CN.

In conclusion, the results arising from noise and NH exposure provide a model of cochlear damage that would combine physical, physiological and metabolic damage at different sites. In particular, the NH model provides strong evidence of involvement of sites downstream from the OHCs.

Chapter 5. The effects of systemic lamotrigine (LTG) on protection against noise induced hearing loss (NIHL)

The experimental basis of this chapter was dependent on the development of a successful model of acute ototrauma as described in Chapter 4. One of the original aims of the experiments presented in that chapter was to develop an acute insult that produced functional deficit that was primarily excitotoxic in character. This would also include any deficit 'downline' subsequent to excitotoxic damage occurring immediately at the post synaptic process. Another aim was also to minimise the contribution due to loss of cochlear structural integrity. The results generated from Chapter 4 showed that exposure to 100dB SPL for 15 minutes provided a marked increase in CN activity during exposure, with post exposure CAP threshold increases and changes in input-output function. These results infer cochlear damage of mixed site origin (OHC, BM, IHC, afferent synapse, CN etc.) but, importantly, an excitotoxic component at the afferent IHC nerve synapse or CN could not be discounted.

The aim of this study was to establish whether neuroprotectants could ameliorate any cochlear functional deficit as a result of this potential excitotoxicity. One such compound with a known neuroprotectant effect both *in vitro* and *in vivo* is lamotrigine (LTG) an established clinically efficacious anticonvulsant developed by GSK (Wiard *et al*, 1995, Smith and Meldrum, 1995). As far as the author is aware, this is the first study investigating both the penetration of LTG into the cochlea and its effects on providing protection against ototrauma, namely acute NIHL.

5.1. Introduction to lamotrigine (LTG) – general mechanisms

The development of LTG is described in a number of reviews (Leach *et al*, 1995, Taylor and Meldrum, 1995, Clare *et al*, 2000 and Palmer and Carter, 2001). Historically, LTG was developed in the 1970s by Burroughs Wellcome as an antifolate, but was subsequently shown to have anticonvulsant properties (Rambeck and Wolf, 1993). This led to further development of LTG, becoming a prototype compound for a family of potentially efficacious anticonvulsants.

It was originally thought that LTG acted by primarily reducing excitatory amino acid release, but it was subsequently shown to act as a voltage dependent Na^+ channel modulator (VDSCM). The current belief is that LTG exerts its anticonvulsant effects through the modulation of voltage-gated Na^+ channels (VGSCs). Previous studies have

demonstrated that LTG produces voltage and use-dependent effects on both recombinant and native rat brain Na^+ channels whilst electrophysiological experiments have indicated that this action is related to selective stabilisation of Na^+ channels in the slow inactivated state as shown simplistically in figure 5.1. The advantage of this mechanism is that LTG can inhibit high frequency firing in *strongly depolarised* neurones but does not interfere with normal synaptic neurotransmission (Xie *et al*, 1995).

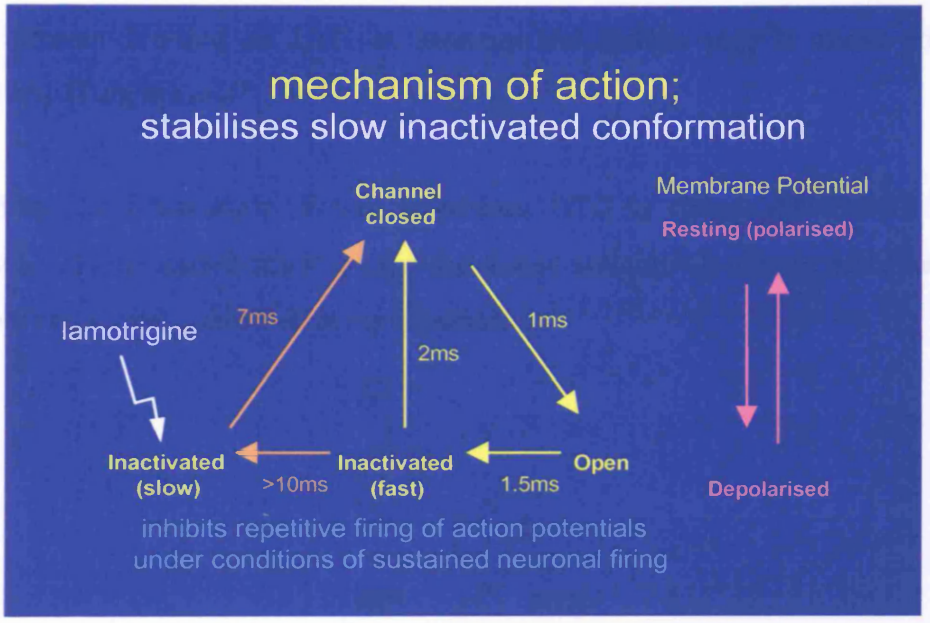


Figure 5.1. A simplistic diagram showing how LTG is believed to act in a highly selective way by stabilising the inactivated Na^+ channel conformation. This results in the inhibition of repetitive firing of action potentials under conditions of sustained neuronal depolarisation. This is believed to inhibit the subsequent release of neurotransmitters downstream (adapted from an internal GSK communication).

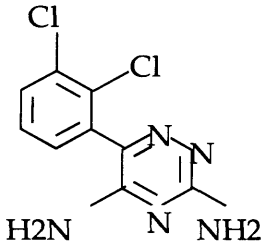
Numerous studies have been performed that clearly show LTG to be a use-dependent blocker of VGSCs as well as inhibiting ischemia-induced 'downline' release of glutamate. Therefore, it has been shown to exhibit neuroprotective as well as anticonvulsant activity (Graham *et al*, 1993, Leach *et al*, 1993, Lustig *et al*, 1992 and Smith and Meldrum, 1993).

Over the last two decades LTG has also been shown to have neuroprotectant properties in stroke as well as being effective in animal models of inflammatory and neuropathic pain, anxiety, Parkinson's disease, substance abuse, hereditary muscle disease, spinal trauma and, more recently, mood stabilisation (Clare *et al*, 2000 and Taylor and Narasimhan, 1997). Clinically, it has also been investigated as a potential tinnitolytic (Simpson *et al*, 1999).

5.2. Chemical structure and properties of LTG

Structurally, LTG is described as a phenyltriazine being prepared by chemical synthesis (figure 5.2). Its universal chemical formula is $C_9H_7Cl_2N_5$, whilst its specific chemical formula is 3,5 diamino-6-(2,3 dichlorophenyl),-1,2,4 triazine. It has a molecular weight of 256.09 Daltons and a pK_a of 5.5. It is poorly soluble in water (<1/1000) but is highly lipophilic enabling it to easily cross both the blood/brain barrier and cell membranes. Plasma protein binding of LTG in humans and guinea pigs is about 55 and 40% respectively (Parsons *et al*, 1995).

Apart from the possession of ring structures, LTG is chemically distinct from other VDSCMs. This includes other voltage dependent anticonvulsants (eg carbamazepine and phenytoin) and local anaesthetics (eg lignocaine).



lamotrigine

Figure 5.2. The chemical structure of lamotrigine (LTG) reproduced with permission from GSK.

5.3.1. Posology and pharmacokinetics of LTG

The initial LTG dose (as Lamictal™) is 25 mg once a day for two weeks, followed by 50mg once a day for two weeks. Thereafter, the dose should be increased by a maximum of 50-100mg every 1-2 weeks until the optimal response is achieved. The usual maintenance dose to achieve optimal response is 100-200 mg/day given once a day or as two divided doses. This dose regimen was also used in the only published data showing the use of LTG in the treatment of tinnitus (Simpson *et al*, 1999) although some patients have required 500 mg/day of Lamictal™ to achieve the desired response as an anticonvulsant (Global Clinical Safety Information, GSK, 2002).

The pharmacokinetics of LTG in man have been concisely reviewed by Richens (1992) Rambeck and Wolf (1993) and Ramsay *et al* (1991). In addition, orally administered LTG has been studied in a number of species including the guinea pig as shown in Table 5.1 (Parsons *et al*, 1995). Good oral bioavailability is demonstrated in animal studies, whilst in humans LTG is completely bioavailable. In most species, the pharmacokinetics

of LTG appear to be dose proportional. Published plasma Cmax values after a clinically relevant dose of 240mg LTG have been quoted as 3.07μg/mL, whilst in the guinea pig this value is 1.2μg/mL following a 4mg/kg dose (Table 5.1). A noticeable difference between guinea pig and humans is that the half-life and clearance rate of LTG is markedly longer in humans than the guinea pig (Parsons *et al*, 1995). This is of relevance in the extrapolation of any experimental studies to man, as the original dosage given in the experimental animal would have to be adjusted to coincide with therapeutically beneficial plasma concentrations.

Elimination of LTG is primarily by hepatic metabolism via N-glucuronidation, prior to elimination by urinary excretion. About 7% of LTG is eliminated without metabolism and there are no known active metabolites in man (Remmel and Sinz, 1991). Tissue: plasma distribution studies in some species (but not the guinea pig) have revealed that LTG is well distributed throughout all tissues and organs. These concentrations are usually 2 to 4 fold higher than in plasma, although in the eye this can extend to 10 fold higher (Parsons *et al*, 1995). In general, excluding the kidney and melanin containing tissues, the rate of clearance is comparable to plasma (Parsons *et al*, 1995). According to current literature, no previous work has been performed investigating LTG levels in inner ear fluids.

Table 5.1. Plasma pharmacokinetics of lamotrigine in laboratory animal species and humans after oral administration (taken from Parsons *et al*, 1995).

Species	Dosage (mg/kg)	T½ (hr)	Cl/F (ml/min/kg)	C_{max} (μg/ml)	V_d/F L/kg)	AUC 0-∞ (μg/ml)
Rat	4	12.5	1.62	1.75	1.79	41.9
Guinea Pig	4	10.3	4.37	1.22	3.91	15.2
Human	240 @	26	0.52	3.07	1.07	118

@ Total human dose
T½ Apparent plasma elimination half life
Cl/F Total apparent plasma clearance/fraction of dose absorbed
C_{max} Maximum concentration of LTG measured in plasma (at time Tmax)
V_d/F Volume of distribution/fraction of dose absorbed
AUC Area under the plasma time concentration curve

5.3.2. Mechanism and sites of action of LTG

To understand the mechanism and sites of action of LTG, a consideration of the general structure and function of VGSCs is necessary. The actions of LTG on high voltage activated (HVA) Ca^{2+} channels and K^+ channels is also discussed. There is a very large literature base on these channels due to their central importance in cell physiology, with

extensive and thorough reviews by Catterall (1992, 1993a-b, 1994) Yang *et al* (1996, 1997) and Marban *et al* (1998).

5.4. General structure of voltage gated Na^+ channels (VGSCs)

The specific Na^+ channels, VGSCs, play a central role in physiology, transmitting depolarizing impulses rapidly throughout neuronal networks, enabling co-ordination of higher processes ranging from locomotion to cognition (Marban *et al*, 1998). There are a large family of VGSCs that reflects their differing functional roles in excitable tissues (Clare *et al*, 2000). Essentially the main groups of VGSCs are those expressed solely in the brain (Types I-IV). There are three other types, two of which are found in the sensory nerves (SN1 and SN2) and one in the peripheral nerves (PN1). Other channels include those found in skeletal, cardiac and glial tissue (Clare *et al*, 2000). In considering the action of LTG on VGSCs in the cochlea or CN, the most probable candidates would be either SN1 or 2. As yet, however, the principal VGSC type(s) have not been identified in CN fibres.

All VGSCs have the same basic structure, illustrated in diagrammatic form in figure 5.3. Each channel consists of four transmembrane polypeptide subunits or domains (DI to DIV). Each domain is made up of six α helices (S1 to S6) connected by intracellular and extracellular polypeptide links. The major α subunit has smaller subunits (β1 to 3) attached to its surface on the extracellular side of the lipid bilayer. These are known to modulate the properties of the α subunit, but are not considered to be necessary for core channel activity. They do, however, appear to be responsible for accelerating channel gating and altering voltage dependence of inactivation and increasing peak current flow (Clare *et al*, 2000). Both extracellular surfaces of the α and β subunits are highly glycosylated. To date, at least 10 α subunits have been identified although structural similarity does exist across the VGSCs (Clare *et al*, 2000).

Ion selectivity for Na^+ is structurally determined by the 'short segment' loops SS1–SS2 residues between S5 and S6 in each domain (figure 5.3). *In situ* these fold over the pore to enhance ion selectivity. In addition, it would appear that the S5 and S6 helices are important for the binding of anticonvulsants, antiarrhythmics and local anaesthetics, as these domains are associated with the inactivation state (Ragsdale *et al*, 1994, 1996 and Wang *et al*, 1998). Interestingly, a single mutation in this region can also lead to a switch in selectivity for Ca^{2+} (Heinemann *et al*, 1992).

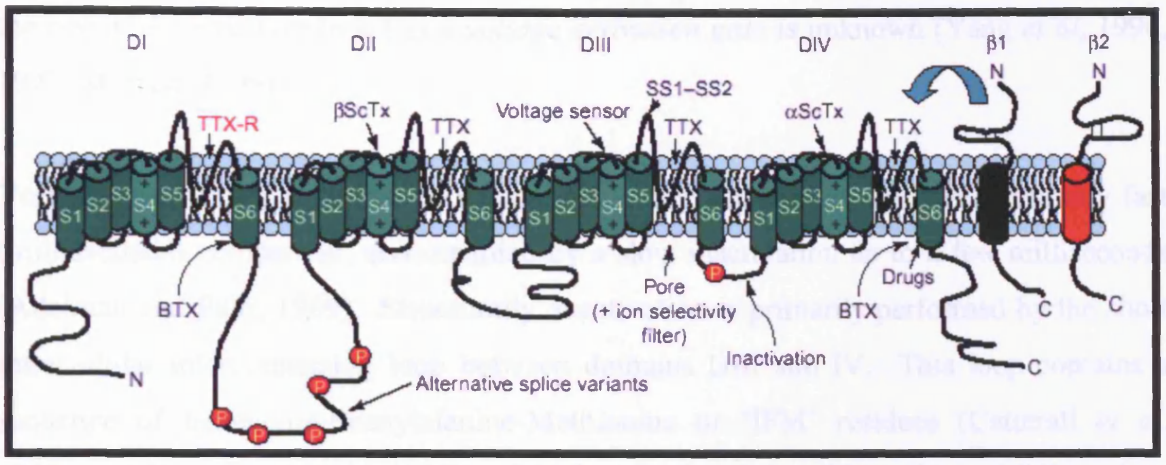


Figure 5.3. The structural domains and binding sites of VGSCs. As inactivation is linked to the S6 helix, drugs that modulate VGSCs are thought to interact in this region. Details shown on DIII are common to all other domains with the exception of inactivation which is specific to DIII-DIV. Taken from Clare *et al*, 2000.

5.4.1. Voltage activation and inactivation of the Na^+ channel

The pioneering series of experiments by Hodgkin and Huxley (1952a, b and c) showed that Na^+ channels existed in three voltage dependent states; resting, active and inactive. In most neurones the resting state potential falls between –60 and –90mV (Rang and Dale, 1991). Activation and opening of the channel depends on there being an adequate depolarisation, typically by about 15 to 20 mV to a threshold voltage of about –50 mV. This leads to the 'all or none' rapid activation and opening of the channel. This depolarisation results from spatial and temporal integration of Na^+ current post synaptically. Depending on channel type, Na^+ current flow typically lasts approximately 1ms.

A fast inactivation then follows, which can last for up to 2ms, during which time the channel is unable to begin generation of another action potential. Together the minimum time over which another action potential can be generated is known as the absolute refractory period or ARP (Catterall, 1993). This is usually followed by a relative refractory period over which the nerve needs to experience a greater depolarisation before it can generate another action potential. The timing of the ARP in the guinea pig CN is relatively short, falling between 0.7 to 0.9ms (Mulheran and Evans, 1986, Mulheran, 1990). This appears to be similar, at least, to the four brain VGSCs (Clare *et al*, 2000).
The voltage activation sensor appears to be within the S4 helix in each of the four channel domains, containing positively charged residues which move in response to depolarisation

(Stühmer *et al*, 1989). The detail of how the movement of the S4 helices is transmitted to the rest of the structure to act as a *voltage activation gate* is unknown (Yang *et al*, 1996, 1997, Marban, 1998).

Voltage inactivation is linked to voltage activation and appears to have a primary fast (milliseconds) component, accompanied by a slow inactivation up to a few milliseconds (Adelman and Palti, 1969). Structurally, inactivation is primarily performed by the short intracellular interconnecting loop between domains DIII and IV. This loop contains a sequence of Isoleucine-Phenylalanine-Methionine or 'IFM' residues (Catterall *et al*, 1993a and Heinemann *et al*, 1992) and is described as acting like a 'hinged lid' to close the intracellular mouth of the pore (West *et al*, 1992). The IFM is thought to act as a hydrophobic latch for the lid (Catterall, 1993a).

5.4.2. Binding site for voltage dependent Na^+ channel modulators (VDSCMs)

The S6 segment of domain IV appears to contain the sites of action for all VDSCMs, in spite of variations in chemical structure for this broad group (Ragsdale, 1994, McPhee *et al*, 1994, 1995, Marban, 1998). The precise details for the interaction of LTG at this site are not known, but mutagenesis experiments combined with electrophysiology suggest that residues F1764 and Y1771 on the DIV S6 segment are key to binding LTG. Substitution at these sites leads to a dramatic reduction of the inhibitory properties of LTG (Liu *et al* 1998). This site is not close to the proposed 'hinged lid' IFM region or the IFM binding site (Eaholtz *et al* 1994).

5.4.3. Voltage and use dependent Na^+ channel modulators (VDSCMs)

The general principles of how Na^+ channel modulators exert their effect have been established in detail, primarily using Chinese Hamster Ovary (CHO) cells. These were transfected with cloned DNA systems expressing Type IIa Na^+ channels (Xie and Garthwaite, 1996). Brain slices from rat have also been used to make recordings from neurones expressing a large proportion of a given type of Na^+ channel cells (Langosch, *et al*, 2000).

Whole cell voltage clamping experiments have been used to investigate the activation/inactivation characteristics of whole populations of Na^+ channels. In these experiments, the cell is experimentally held at a set or holding voltage (V_h) above and below the usual resting potential. This showed that with V_h at increasingly lower

depolarising potentials, proportionately more channels existed in the inactivated state (Ruben *et al*, 1992).

Experiments of this kind have also been used to demonstrate the voltage dependence of the effect of VDSCMs including LTG. When concentration response curves were constructed for whole cell Na^+ channel currents (V_h values of either –90 or -60mV) LTG IC_{50} values of 641μM and 56μM respectively were obtained. This showed that LTG acted preferentially on nerve fibres held at –60mV due to a greater number of channels being held in the inactivated state at this voltage (figure 5.4A, taken from GSK internal report, SR1998/0004/00).

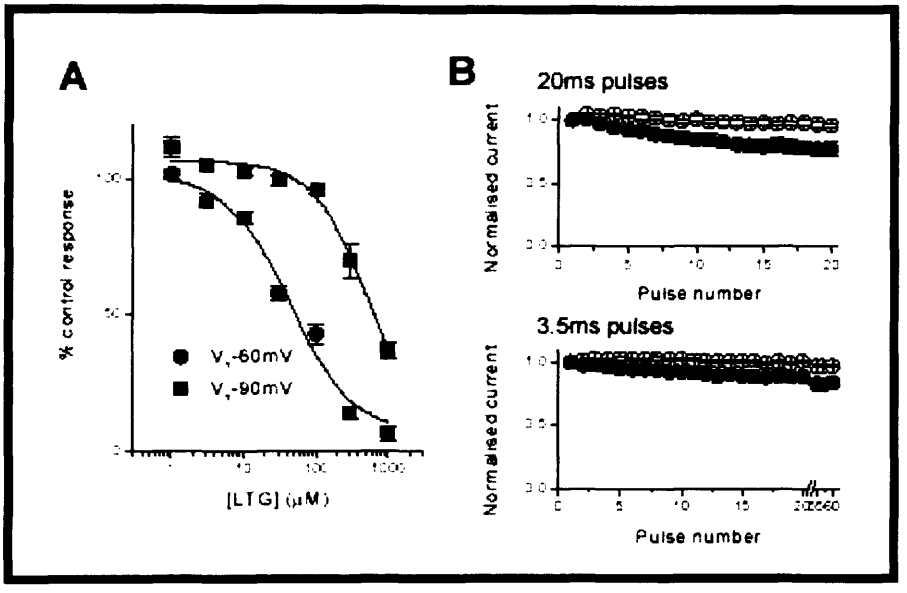


Figure 5.4. Concentration-response curves of inhibition by LTG on **A)** recombinant human type IIA Na^+ channels. Holding potentials (V_h) of –90 and –60mV were applied followed by test pulses to 0mV to elicit currents. LTG demonstrated a voltage-dependent action on recombinant type IIA Na^+ channels showing half inhibitory concentrations (IC_{50}) of 641μM and 56μM for V_h values at –90 and -60mV respectively.
B) Use-dependent effect of LTG on recombinant human type IIA Na^+ channels. Under control conditions very little (<10%) 'run-down' was observed between the first and twentieth pulse. In the presence of LTG (100μM) significantly greater use-dependence was observed for both pulse durations, but were greatest when long (20ms) pulses were applied (GSK internal report, SR1998/0004/00).

Use dependent experiments utilise a train of depolarising pulses (from -90mV to 0mV). In experiments with LTG (100μM) the system was stimulated with a train of depolarising pulses of varying duration, between 3.5 to 20ms. Inhibition of type IIA Na^+ channels increased as the pulse duration increased (figure 5.4B).

5.4.5. LTG and VDSCM recovery from inactivation phase

Presumably it is the prolonged recovery from inactivation that underlies the actual mechanism of action of LTG in reducing Na^+ current into the neurone, as shown in the

simplistic diagram of activation and inactivation (figure 5.1). This increased time delay for recovery would mean that the probability of a neurone discharging after a previous action potential, in which LTG had bound, would then be reduced.

5.5. Effects of LTG on Ca^{2+} and K^+ conductance

Because of the similarities in structure between the main voltage dependent cationic ion channels (Catterall, 1993a-b, Godfraind and Govoni, 1995) it was important to establish if LTG was also acting at these sites. Voltage dependent Ca^{2+} channels in sensory neurons were originally classified into three main types; L, T and N according to activation and inactivation ranges and time course of current decay (Nowycky *et al*, 1985). L-Type channels activate at high membrane potentials (high voltage activated, or HVA) and inactivate slowly. T-Type channels activate at low membrane potentials (low voltage activated, or LVA) and inactivate quickly. N-type channels also activate at high membrane potentials but inactivate more slowly than T-Type but much faster than L channels. Three other Ca^{2+} current types have since been identified namely P, Q and R (Catterall, 2000). Although L and T-type channels are present in a wide range of cell types, N, P, Q and R-type channels are most prominent in neurons (Catterall, 2000).

A new nomenclature for voltage gated Ca^{2+} channels has recently been proposed using the principal permeating ion and the principal physiological regulator, represented as Ca_v, where 'v' is voltage (Ertel *et al*, 2000). The Ca_v1 family ($Ca_v1.1$ to $Ca_v1.4$) includes channels containing α_{1S}, α_{1C}, α_{1D}, and α_{1F} subunits mediating L-type Ca^{2+} currents. The Ca_v2 family ($Ca_v2.1$ to $Ca_v2.3$) includes channels containing α_{1A}, α_{1B} and α_{1E} subunits which mediate P/Q-type, N-type, and R-type Ca^{2+} currents, respectively. The Ca_v3 family ($Ca_v3.1$ to $Ca_v3.3$) includes channels containing α_{1G}, α_{1H} and α_{1I} which mediate T-type CA^{2+} currents. To date, receptor subtypes identified in the cochlea include $Ca_v1.3$ (α_1 1.3) and $Ca_v1.4$ (α_1 1.4) mediating the HVA L-type Ca^{2+} channels, $Ca_v2.1$a and b (α_1 2.1) mediating P/Q-types and $Ca_v2.3$b (α_1 2.3) mediating R-type channels (Ertel *et al*, 2000).

High Voltage Activated L and N-type Ca^{2+} channels have shown weak to moderate (2-40%) voltage dependent effects *in vitro* over 20μM to 1mM concentrations of LTG (figure 5.5, GSK internal report, SR1998/0004/00). It is therefore conceivable that, *in vivo*, LTG may secondarily exert some of its neuroprotective effects via HVA conductances, especially if these channels were activated for a prolonged period in a depolarised state (Wang *et al* 1996, Stefani *et al*, 1996 and Von Wegerer *et al*, 1997). In

addition, work performed by Hainsworth *et al* (2001) showed that *in vitro* experiments on isolated cortical neurones resulted in LTG inhibiting approximately 34% of the recorded HVA Ca^{2+} current (IC_{50} values of between 7.5 and 9μM, depending on the pH).

LTG only appears to have weak, non-voltage dependent inhibitory effects on K^+ current, with concentrations of LTG up to 100μM (Xie and Hagan 1998, GSK internal report SR1998/0004/00). Delayed rectifier K^+ currents (I_K) currents of hippocampal neurones were evoked by depolarising pulses to +30mV from a holding potential of –90mV. Both A-type (I_A) and I_K K^+ channel currents were also examined from DRG neurones (GSK internal report, SR1998/0004/00). LTG up to 100μM, caused less than 10% inhibition of the I_K current and had no significant effects (1±2% and 1±3%) for I_K and I_A respectively (figure 5.6).

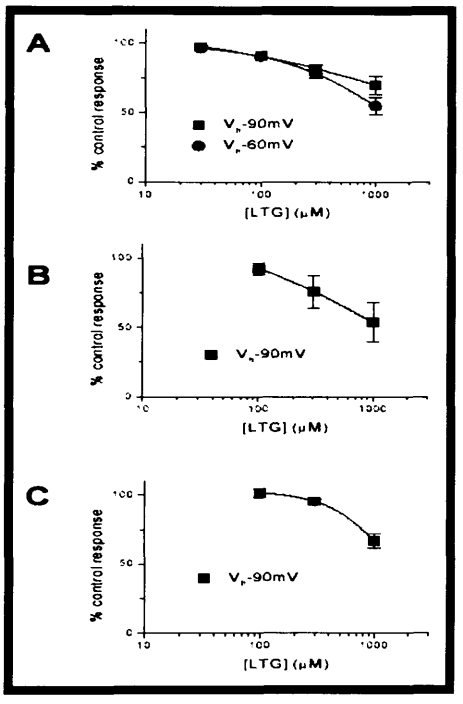


Figure 5.5. Concentration response curves constructed for LTG on (**A**) recombinant N-type Ca^{2+} channels, (**B**) T-type Ca^{2+} channels of TT-cells and (**C**) high voltage activated (HVA) Ca^{2+} channels of DRG neurones. When a V_h of –90mV was used LTG, up to 1mM, caused <50% inhibition against all the Ca^{2+} channels tested. For the cloned N-type Ca^{2+} channels (A), a V_h of –60mV was also applied and LTG produced a slightly greater inhibition, suggesting some degree of voltage-dependency (GSK internal report, SR1998/0004/00).

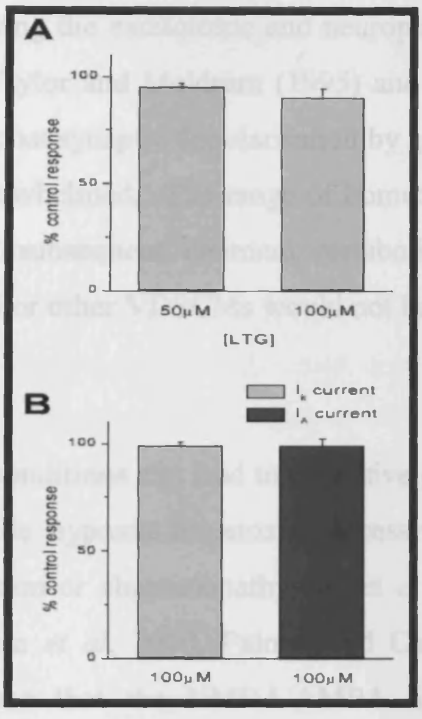


Figure 5.6. A) Delayed rectifier K^+ currents (I_K) currents of hippocampal neurones were evoked by depolarising pulses to +30mV from a holding potential of –90mV. LTG, up to 100μM, caused less than 10% inhibition of the I_K current. **B)** Both A-type (I_A) and I_K K^+ channel currents were examined from DRG neurones. LTG, up to 100μM, had no significant effects (1±2% and 1±3% for I_K and I_A, respectively, n=4). Taken from GSK internal report, SR1998/0004/00.

5.6. LTG and VDSCMs in neuroprotection - a provisional mechanism

Originally, LTG and other VDSCMs were considered to be of principle therapeutic use in epilepsy and pain (see reviews by Taylor and Meldrum, 1995 and Clare *et al*, 2000). Work throughout the 1990s has also showed that VDSCMs had therapeutic potential in experimental models of ischaemia and stroke (Leach, 1993, Smith *et al*, 1993, Graham *et al*, 1994, Sun and Faden, 1995, Kawaguchi and Graham, 1997, Carter, 1998). These models included both global and localised ischaemia *in vivo*. *In vitro* models using brain slice or tissue cultures, employed glutamate agonists or oxygen/glucose deprivation to compromise normal neuronal function (Tasker *et al*, 1992, Stys *et al,* 1992, Fern *et al,* 1993, Weber and Taylor, 1994, Pisani *et al*, 2001).

Both these *in vivo* and *in vitro* models have clear parallels with the noise and noise/hypoxia model developed and described in Chapters 3 and 4. Noise stimulus acts to place an acute heavy metabolic and functional demand on the system. The moderate hypoxia augments this challenge by restricting the ability of the auditory periphery to deal effectively with this demand.

The general features underlying the excitotoxic and neuroprotective effects of VDSCMs have been summarised by Taylor and Meldrum (1995) and are illustrated in figure 5.7. Under normal conditions of post synaptic depolarisation by glutamate, the neurone and its constituent parts are not overwhelmed. The range of homeostatic mechanisms available to offset excitotoxicity and subsequent neuronal metabolic overload are operational. Under these conditions, LTG or other VDSCMs would not be expected to exert any effect on Na^+ channel function.

Various pathophysiological conditions can lead to excessive glutamate levels persisting at receptor sites. These include hypoxia or anoxia, excessive stimulation by the input pathway, metabolic dysfunction or channelopathy (Billet *et al*, 1969, Pujol *et al*, 1993, Puel *et al*, 1994, 1995, Clare *et al*, 2000, Palmer and Carter, 2001). The continued presence of glutamate means that the NMDA/AMPA ligand gated influx of Na^+, accompanied secondarily by Ca^{2+}, leads to substantial and prolonged depolarisation.

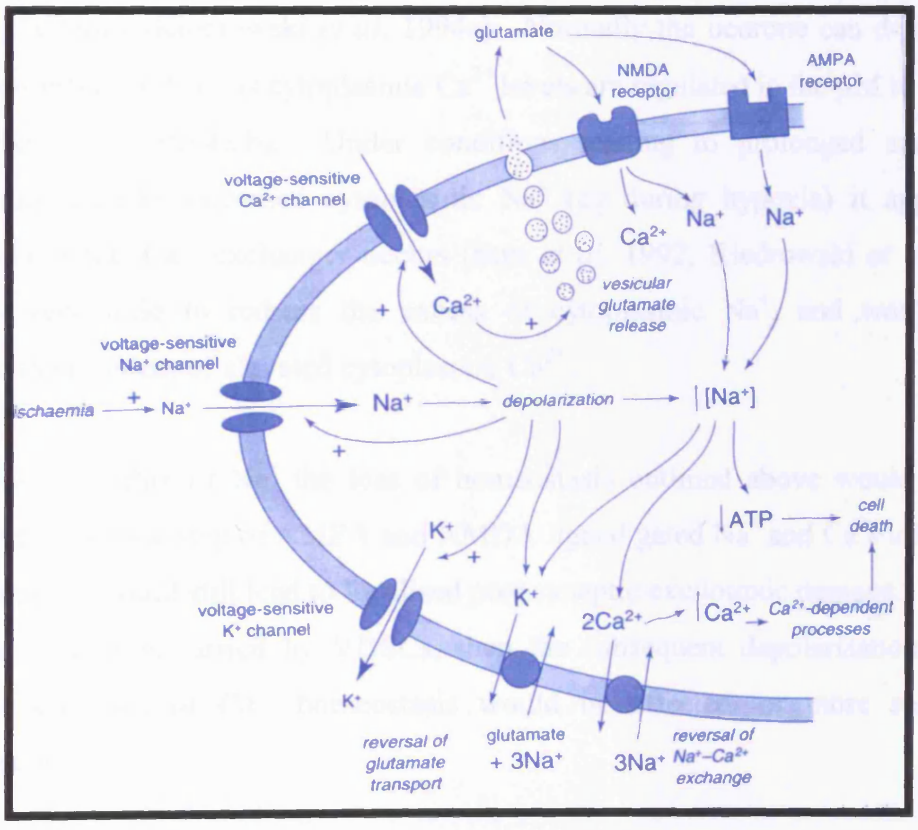


Figure 5.7. A schematic representation of the role of Na^+ and Ca^{2+} channels during an ischaemic episode, highlighting both neurotoxic and excitotoxic processes (Taken from Taylor and Meldrum, 1995).

This ionic movement also leads to osmotic movement of water, leading to swelling of the neuron. The depolarisation leads to continued rebound activation of VDSCs and further

influx of Na^+. This adds to the increasing cytoplasmic pool of Na^+ that begins to defeat the normal homeostatic mechanisms in a number of ways.

Adenosine triphosphate (ATP) is essential for maintaining the Na^+/ K^+ balance at normal levels of activity. The excessive demand on ATP then leads to its depletion so it is even less capable of dealing with Na^+ overload. This loss of ATP means that other vital energy requiring activities are compromised. One of the most important is Ca^{2+} homeostasis. It is well recognised that if left unchecked, severe loss of Ca^{2+} homeostasis can lead to cell death (Bruno *et al*, 1993, Pujol *et al*, 1993). With the moderate level of noise insult described in chapters 3 and 4, excessive chronic depletion of ATP was considered less likely to occur.

However, cytosolic levels of Ca^{2+} would be expected to be elevated further by interference with the normal neuronal mechanism for Ca^{2+} extrusion. At rest, this involves the operation of a Na^+-Ca^{2+} exchanger that inwardly transports 3 Na^+ ions to extrude 2 Ca^{2+} ions (Kiedrowski *et al*, 1994a). Normally the neurone can deal with the very modest influx of Na^+, as cytoplasmic Ca^{2+} levels are regulated in the μM to nM range (Kiedrowski *et al*, 1994a-b). Under conditions leading to prolonged and marked depolarisation due to increased cytoplasmic Na^+ (eg during hypoxia) it appears that reversal of the Na^+-Ca^{2+} exchanger occurs (Stys *et al*, 1992, Kiedrowski *et al*, 1994a). This does very little to redress the excess of cytoplasmic Na^+, and would further exacerbate the problem of elevated cytoplasmic Ca^{2+}.

In blocking the influx of Na^+ the loss of homeostasis outlined above would be partly ameliorated. The first step of AMPA and NMDA ligand gated Na^+ and Ca^{2+} influx is not affected, and this could still lead to localised post synaptic excitotoxic damage. However, where Na^+ current is carried by VDSCs, then the subsequent depolarisation, osmotic movement and loss of Ca^{2+} homeostasis would be affected, or more specifically, neuroprotective.

There is also evidence that at subsequent synaptic transmission, there is an exacerbated release of glutamate that is not Ca^{2+} dependent (Szatkowski and Attwell, 1994, Taylor *et al*, 1995). This results from a reversal of the glutamate/ Na^+ co-transporter that normally operates to rapidly remove glutamate from the synaptic cleft. This transport mechanism is

also likely to operate in the IHCs and adjacent supporting cells to remove glutamate form the synapse (Pujol *et al*, 1993).

It is possible that all of these features apply in the noise and noise/hypoxia acute ototrauma model. The novel experimental utilisation reported for the first time here of the well characterised VDSCM, LTG, enables further dissection of the relative involvement of sites in the auditory periphery following the insult.

5.7. Aims of LTG studies

The aims of this study were essentially three-fold:

1. To study the pharmacokinetic profile of orally administered LTG in perilymph and plasma obtained from albino guinea pigs. These data would provide evidence for LTG entry into the cochlear duct and allow direct comparison against plasma LTG levels. No electrophysiological recordings were performed in this experiment.

2. Based on the pharmacokinetic studies above, establish the effects of LTG on normal auditory function using direct electrophysiological measurements (CAP and ESAC). If LTG affected normal cochlear function, then assessment of any potential cochlear protection would be compromised.

3. Using the results collected above, perform 15 minute noise exposure studies (as described in Chapter 4) around the time of LTG C_{max} within the cochlea, thereby providing the best opportunity for protection against NIHL.

5.8. Justification of the LTG dose level and formulation

Data generated within GSK using the rat has shown an effective oral dose (ED_{50}) of LTG to be 6.8mg/kg in the supramaximal electroshock (MES) model. This value was sometimes slightly higher but on the whole this was a representative figure.

Published area under the plasma time concentration curve data (Parsons *et al*, 1995) showed apparently greater absorption in the rat compared to the guinea pig (41.9 cf.15.2μg/ml in the guinea pig). Based on this information, it was felt that to provide the best opportunity for obtaining adequate plasma/perilymph LTG concentration data, an oral dose of 20mg/kg in the guinea pig was suitable. In addition, the systemic side effect

profile from this dose level was likely to be negligible. No other dosage levels of LTG were selected due to time constraints and animal usage.

Lamotrigine (isethionate salt) synthesised by Medicinal Sciences, GSK, Stevenage, was dissolved in sterile water for irrigation to produce a 4mg/mL solution (1g of base was equivalent to 1.4925g of isethionate salt). A dose volume of 5mL/kg was employed yielding a total dose of 20mg/kg. These dosing parameters remained standard for all LTG treated animals. All solutions were prepared fresh on the day of dosing and stored at 2-8°C in the dark between dosing sessions.

5.9. Experimental Methods

Animal supply, husbandry, diet and drinking water, surgical preparation and sedation (regimen 3) are all detailed in the relevant sections in Chapter 2.

5.9.1. Plasma and perilymph collection for lamotrigine (LTG) analysis

Prior to any electrophysiological or noise exposure studies with LTG, plasma and perilymph samples were taken from 3 animals/timepoint at 1, 2, 5, 10 and 15 hours following a single oral dose of 20mg/kg LTG. This ensured that subsequent studies were performed at the optimal time of LTG exposure. Two untreated control animals were also sampled for comparison. Full details of terminal blood and perilymph sampling, and LTG analysis are described in Chapter 2, sections 2.16 and 2.17.

5.9.2. LTG control and noise exposure studies

These experiments were conducted in two parts as shown in Table 5.2. Firstly, prior to any noise exposure, guinea pigs were assessed to ensure no loss of cochlear function from systemic administration of 20mg/kg LTG alone. The optimal time of testing of 2 hours after dosing was established from the pharmacokinetic experiment described in section 5.9.1. Suitable untreated controls were used as comparators. The second part comprised animals exposed to 100 dB SPL band pass noise over 5 to 20 kHz, for 15 minutes as detailed in previously. This was performed in both the presence and absence of LTG. Plasma and perilymph samples were also collected as terminal procedures.

Artificial perilymph was applied to the round window membrane for at least 15 minutes prior to insult. Both CAP and ESAC recordings were taken at the start and end of this period as described in chapter 2, with EDAC recordings taken at 5 minute intervals during noise exposure. At least one more ESAC measurement was made during a short recovery

period of up to 25 minutes. In the case of the controls, artificial perilymph was in place for 60 minutes to ensure no adverse effects on the cochlea as a result of the solution application.

Table 5.2. Study design

	Group Number	Treatment	Noise exposure (100 dB SPL)	Number of animals
Part 1	1	Control (No treatment)	No	7
	2	LTG (20mg/kg oral[#])	No	11
Part 2	3	Noise	Yes	7
	4	LTG (20mg/kg oral[#]) + noise	Yes	7

[#] Oral administration via gavage (single dose) – Administered approximately 2 hours prior to noise insult.

5.10. Results

As in previous chapters, because of unequal numbers of animals across treatment groups, carrying out within group and across group one way ANOVA was considered to be the most appropriate statistical analysis. Across group analysis was carried out to establish the effects of noise/LTG compared to noise alone. The lack of pre LTG CAP/ESAC data in the LTG treated animals, means that statistical inference between time controls and LTG alone was partly limited. Statistical results are summarised in Appendix 1.

5.10.1. The pharmacokinetics of LTG in plasma and perilymph

Lamotrigine (LTG) was detected in plasma and perilymph, indicating good systemic exposure and penetration of the inner ear (figure 5.8). Levels of total LTG detected in perilymph were approximately twice those seen in plasma with a mean maximum concentration (C_{max}) in perilymph of 6.1±0.8μg/mL compared to 3.6±0.8μg/mL in plasma. Mean time of maximum concentration (T_{max}) for perilymph was 2 hours compared to 1 hour for plasma. These results show the preferential uptake of LTG by the cochlea.

Mean plasma and perilymph LTG levels remained relatively high after the 15 hour sampling period at 1 and 3.2μg/mL respectively, indicating comparable elimination rates and no evidence to suggest accumulation in cochlear fluids. No overt signs of toxicity were seen throughout the experiment in any animal. Based on these data, all future control LTG and noise exposed LTG electrophysiological data were collected

approximately 2 hours after dosing, equivalent to an approximate C_{max}. This provided the optimal opportunity for observing drug action during experiments.

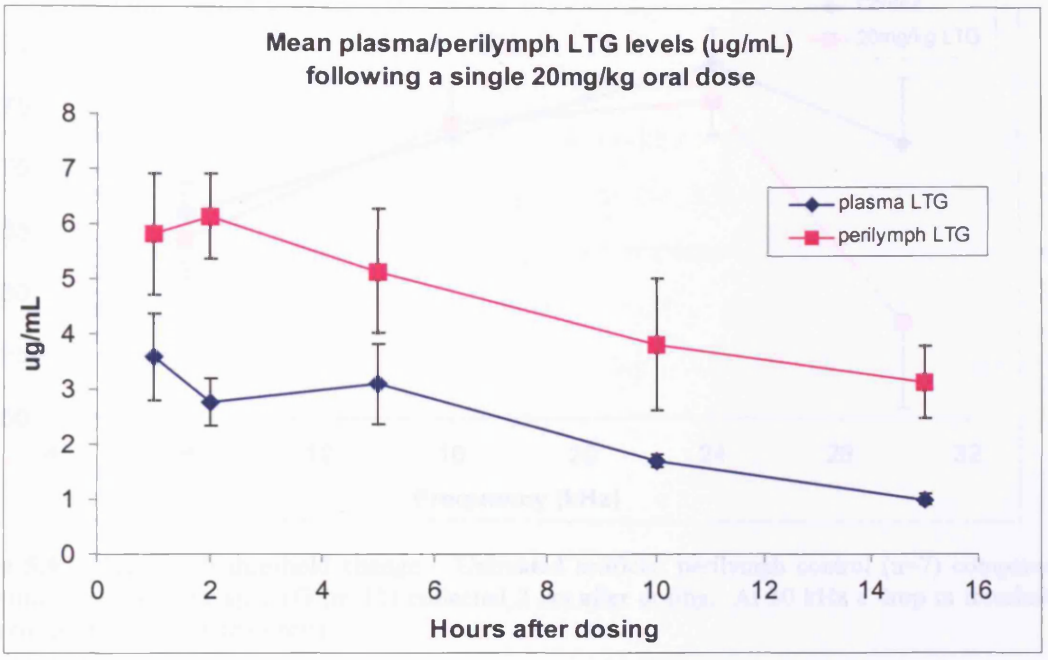


Figure 5.8. Plasma and perilymph LTG levels after a single 20mg/kg oral dose (n=3/timepoint/group).

5.10.2. Effects of LTG on CAP threshold - Control versus 20mg/kg LTG

In the absence of noise exposure, mean CAP threshold data were similar for control and LTG treated groups at 8, 16 and 24 kHz (figure 5.9). Mean values of 66.4±2.53, 72.8±4.26 and 78.6±2.87 dB SPL for controls compared to 64.4±3.03, 73.9±1.68 and 75.6±2.87 dB SPL for the LTG treated group were obtained at 8, 16 and 24 kHz respectively.

A non-significant increase ($p=0.129$) in threshold at 30 kHz was noted for the LTG treated group compared to control. These values were 57.8±7.0 and 72.2±5.24 dB SPL for LTG and control respectively. In the LTG group, three animals showed particularly lower than normal threshold values at 30 kHz, over the range of 26 to 36 dB attenuation, which contributed to this result.

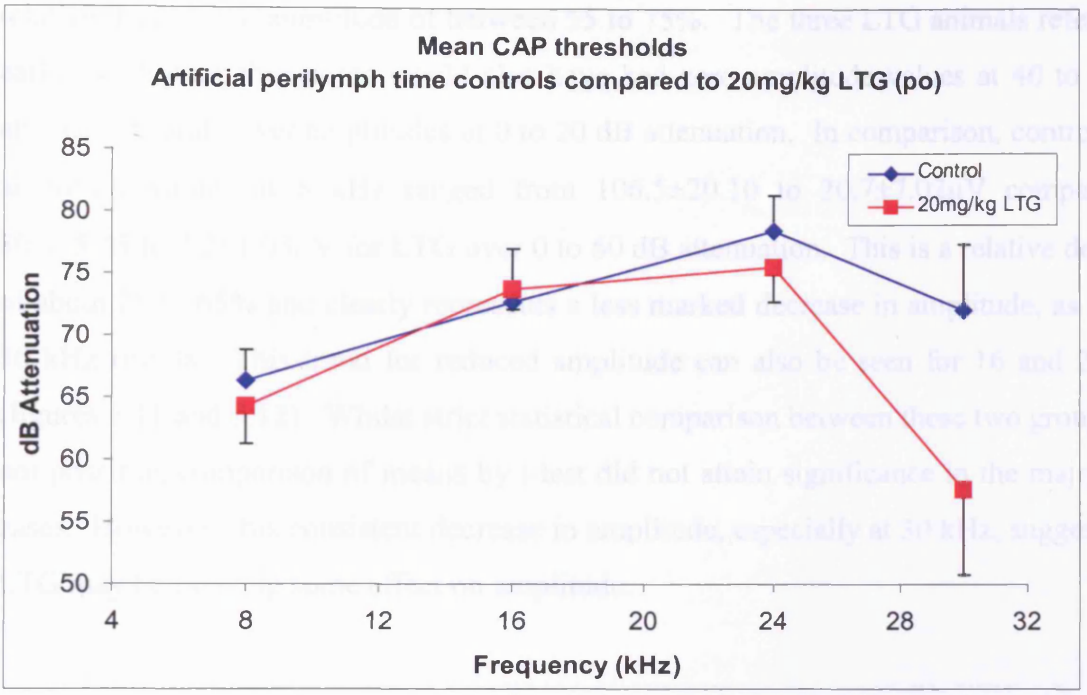


Figure 5.9. Mean CAP threshold change – Untreated artificial perilymph control (n=7) compared to a single oral dose of 20mg/kg LTG (n=11) collected 2 hrs after dosing. At 30 kHz a drop in threshold was observed (p=0.129, Student's t test).

5.10.3. Effects of LTG on CAP N_1 amplitude - Control versus 20mg/kg LTG

Because no pre-LTG treatment data were collected, direct statistical comparison with the artificial perilymph control group over time was not possible. However, based on threshold data where no marked effects were seen between the two groups, visual comparison between the control and LTG groups is presented below. It was immediately apparent that lower amplitude values for LTG treated animals were noted at all four frequencies tested, especially at 0dB attenuation, with a trend towards lower amplitude values at all levels of attenuation. The most affected frequency appeared to be 30 kHz.

This is of interest especially as lower threshold values were noted at this frequency compared to the control group. Figures 5.10 to 5.13 show the responses seen at 8, 16, 24 and 30 kHz respectively. It is relevant to note at this point that the large standard error bars shown for the artificial perilymph control group reflects higher than expected amplitude values for 2 animals within this group at all frequencies. For example, at 16 kHz values of 350 and 370 μVolts, 0dB attenuation were attained, compared to values between 100 and 180μVolts in the remaining animals.

Control CAP amplitude values at 30 kHz ranged from 115.6±40.74 to 22.1±9.09μV compared to 50.7±7.97 to 5.1±2.39μV for LTG over 0 to 60dB attenuation, representing a

relative decrease in amplitude of between 55 to 75%. The three LTG animals referred to earlier with poor thresholds would also have had zero amplitude values at 40 to 60 dB attenuation, and lower amplitudes at 0 to 20 dB attenuation. In comparison, control CAP amplitude values at 8 kHz ranged from 106.5±20.10 to 20.7±7.02μV compared to 80.6±5.45 to 7.2±1.03μV for LTG over 0 to 60 dB attenuation. This is a relative decrease of about 25 to 65% and clearly represents a less marked decrease in amplitude, as for the 30 kHz results. This trend for reduced amplitude can also be seen for 16 and 24 kHz (figures 5.11 and 5.12). Whilst strict statistical comparison between these two groups was not possible, comparison of means by t-test did not attain significance in the majority of cases. However, this consistent decrease in amplitude, especially at 30 kHz, suggests that LTG may be exerting some effect on amplitude.

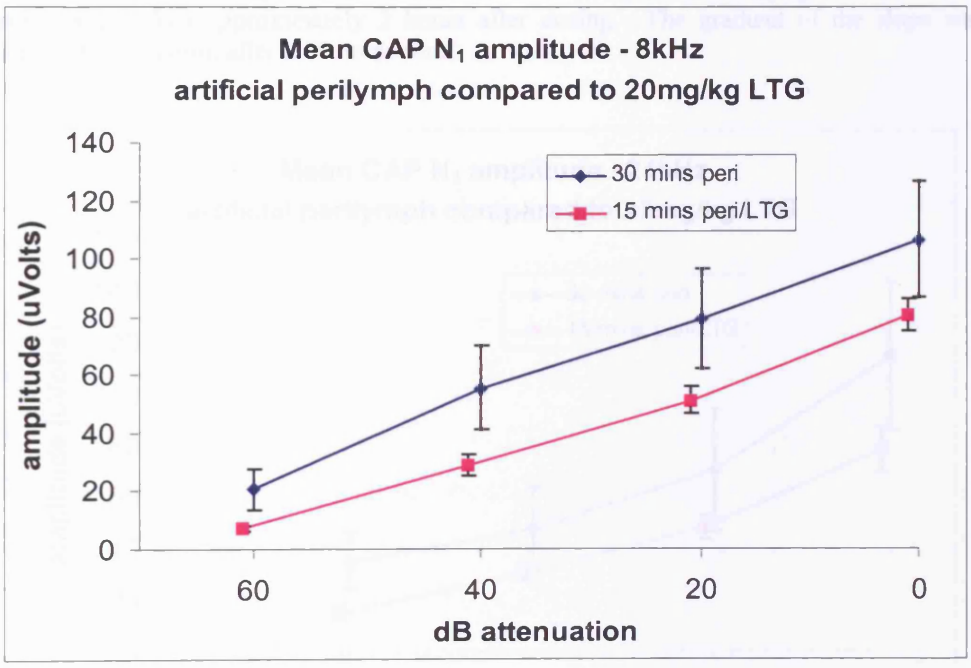


Figure 5.10. Mean CAP N_1 amplitude at 8 kHz – control (n=7) versus 20mg/kg LTG (n=11). The LTG measurements were taken approximately 2 hours after dosing. Overall, the gradient of the slope was similar with a slight decrease in amplitude noted after LTG treatment.

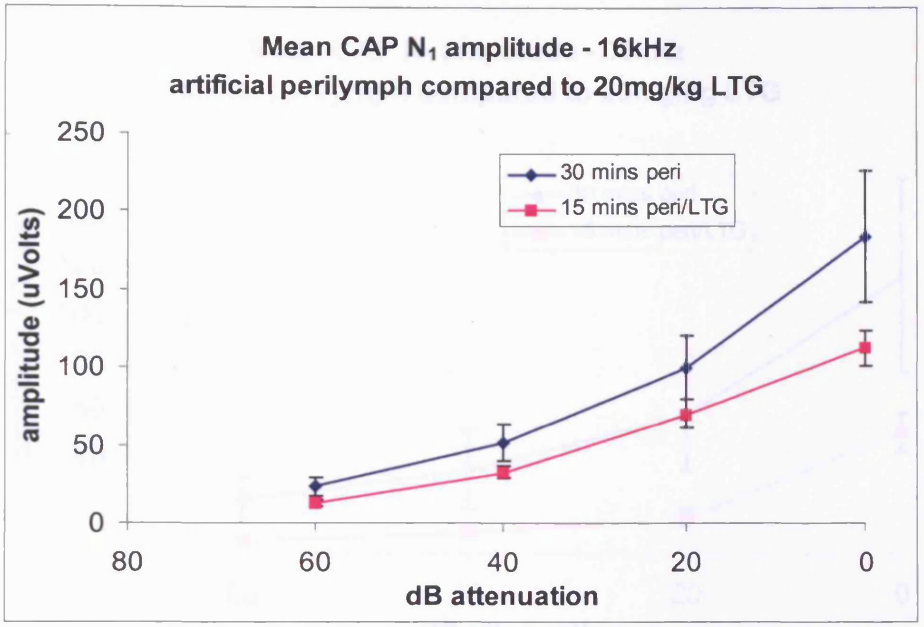


Figure 5.11. Mean CAP N_1 amplitude at 16 kHz – control (n=7) versus 20mg/kg LTG (n=11). The LTG measurements were taken approximately 2 hours after dosing. The gradient of the slope was slightly decreased at 0dB attenuation after LTG treatment.

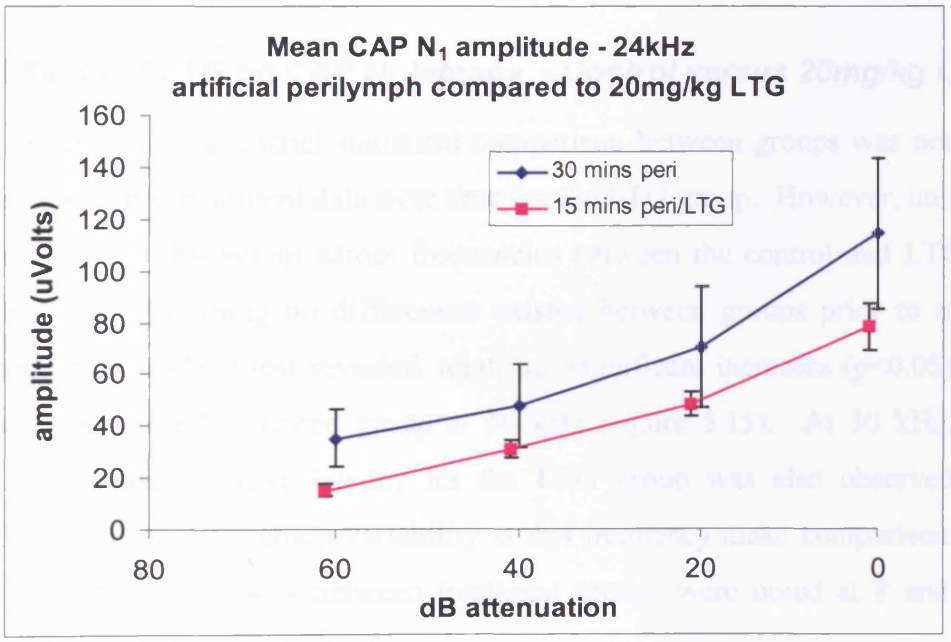


Figure 5.12. Mean CAP N_1 amplitude at 24 kHz – control (n=7) versus 20mg/kg LTG (n=11). The LTG measurements were taken approximately 2 hours after dosing.

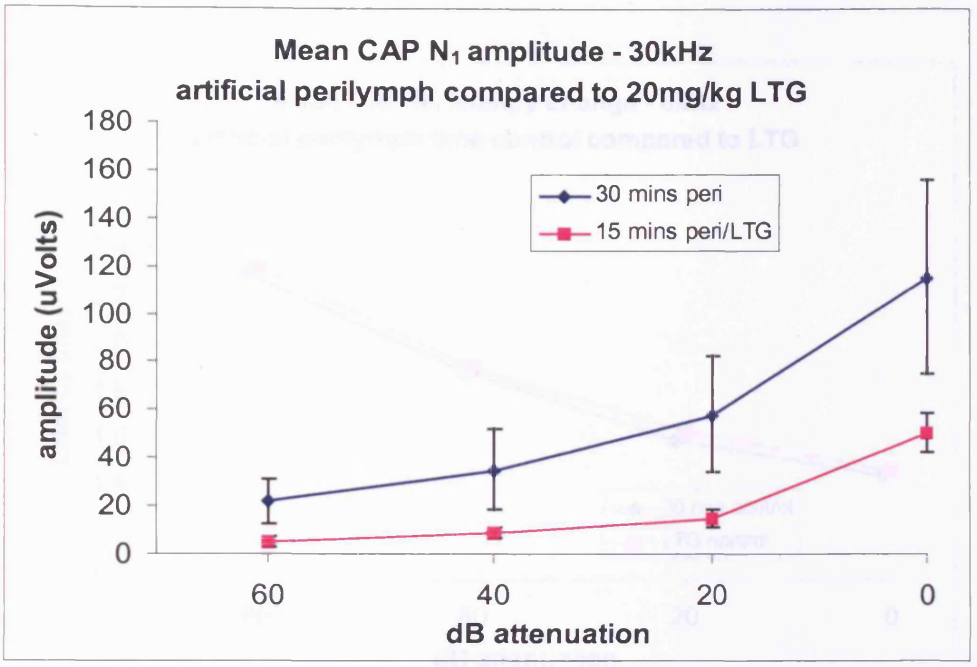


Figure 5.13. Mean CAP N_1 amplitude at 30 kHz – control (n=7) versus 20mg/kg LTG (n=11). The LTG measurements were taken approximately 2 hours after dosing. Overall, the gradient of the slope was similar but a marked decrease in amplitude was noted after LTG treatment.

5.10.4. Effects of LTG on CAP N_1 latency - Control versus 20mg/kg LTG

As for N_1 CAP amplitude, strict statistical comparison between groups was not feasible due to the lack of pre-treatment data over time for the LTG group. However, unlike wave amplitude, no clear distinction across frequencies between the control and LTG groups was established. Assuming no differences existed between groups prior to treatment, comparison of means by t-test revealed small but significant increases ($p<0.05$) in CAP wave latency in the LTG treated group at 16 kHz (figure 5.15). At 30 kHz, a trend towards an increase in wave latency for the LTG group was also observed, but as explained previously, inter group variability at this frequency make comparison difficult (figure 5.17). No differences between treatment groups were noted at 8 and 24 kHz (figures 5.14 and 5.16).

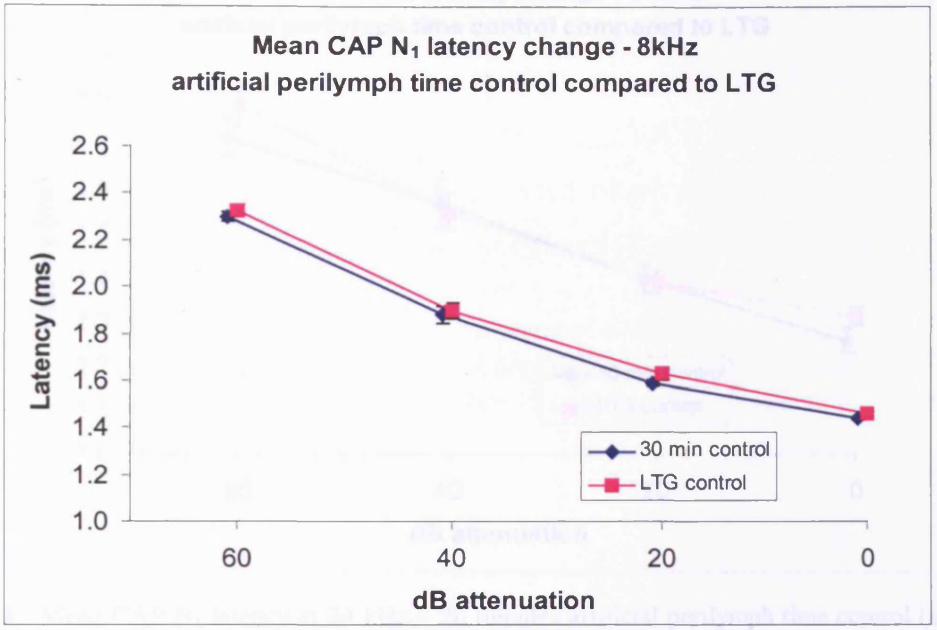


Figure 5.14. Mean CAP N_1 latency at 8 kHz – 30 minutes artificial perilymph time control (n=7) versus 20mg/kg LTG (n=11). For the LTG group, measurements were taken approximately 2 hours after dosing.

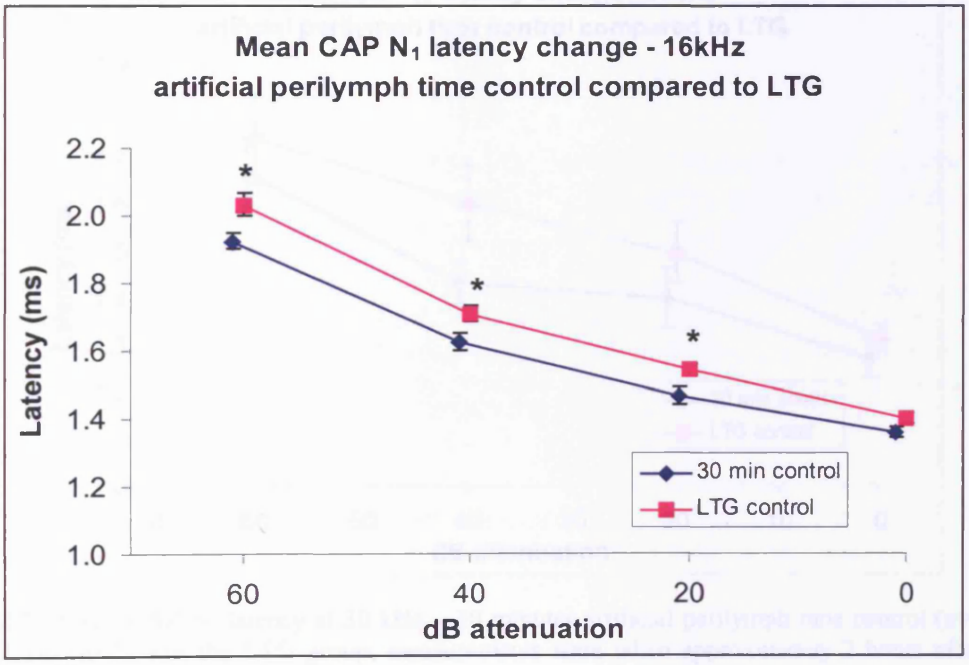


Figure 5.15. Mean CAP N_1 latency at 16 kHz – 30 minutes artificial perilymph time control (n=7) versus 20mg/kg LTG (n=7). For the LTG group, measurements were taken approximately 2 hours after dosing. Significant increases in wave latency for the LTG group compared to control was observed at all levels of attenuation with the exception of 0dB (*$p<0.05$, t-test).

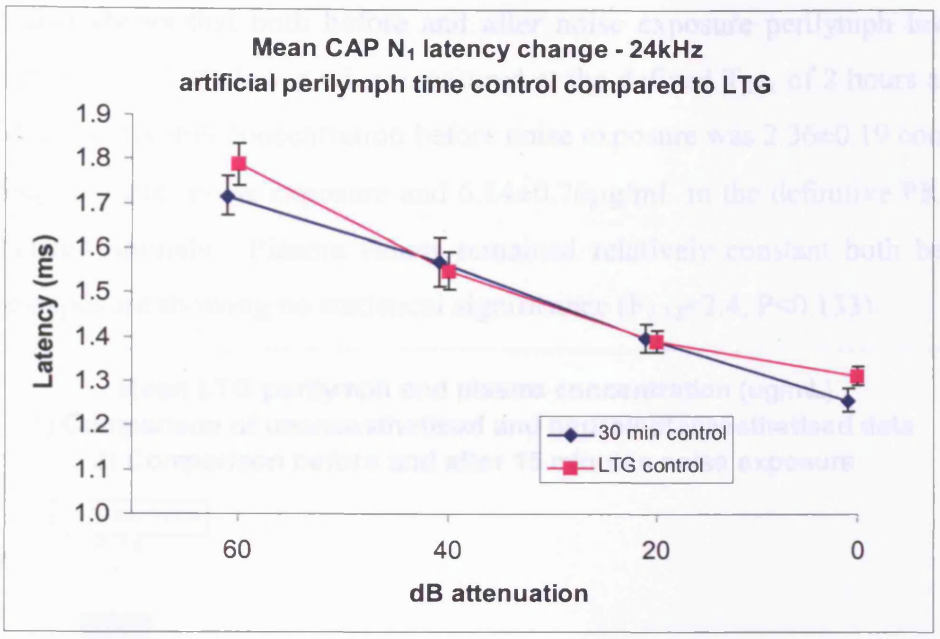


Figure 5.16. Mean CAP N_1 latency at 24 kHz – 30 minutes artificial perilymph time control (n=7) versus 20mg/kg LTG (n=7). For the LTG group, measurements were taken approximately 2 hours after dosing.

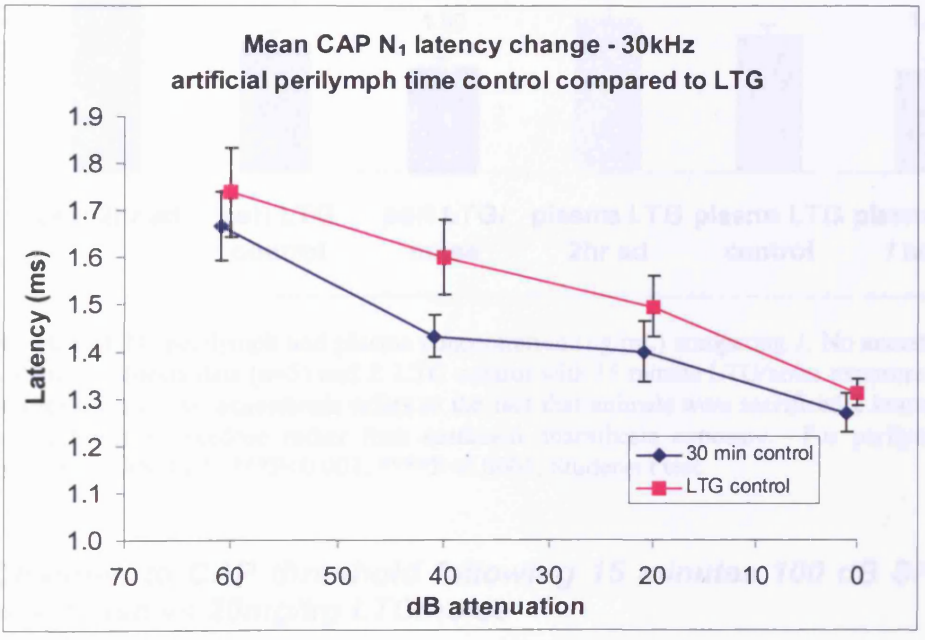


Figure 5.17. Mean CAP N_1 latency at 30 kHz – 30 minutes artificial perilymph time control (n=7) versus 20mg/kg LTG (n=7). For the LTG group, measurements were taken approximately 2 hours after dosing. Inter group variability make interpretation difficult as shown by the large standard error bars.

5.10.5. The pharmacokinetics of LTG in plasma and perilymph in unanaesthetised and neuroleptanaesthetised animals following noise exposure

Data collected from LTG treated neuroleptanaesthetised animals used in this noise exposure study revealed statistically significantly lower perilymph levels when compared to the unanaesthetised control data in section 5.10.1 (figure 5.8) as shown in figure 5.18.

This plot also shows that both before and after noise exposure perilymph levels were approximately 60 to 70% lower when measured at the defined T_{max} of 2 hours after LTG dosing. Mean perilymph concentration before noise exposure was 2.36±0.19 compared to 1.89±0.19µg/mL after noise exposure and 6.14±0.76µg/mL in the definitive PK study in unanaesthetised animals. Plasma values remained relatively constant both before and after noise exposure showing no statistical significance ($F_{2,12}$=2.4, P<0.133).

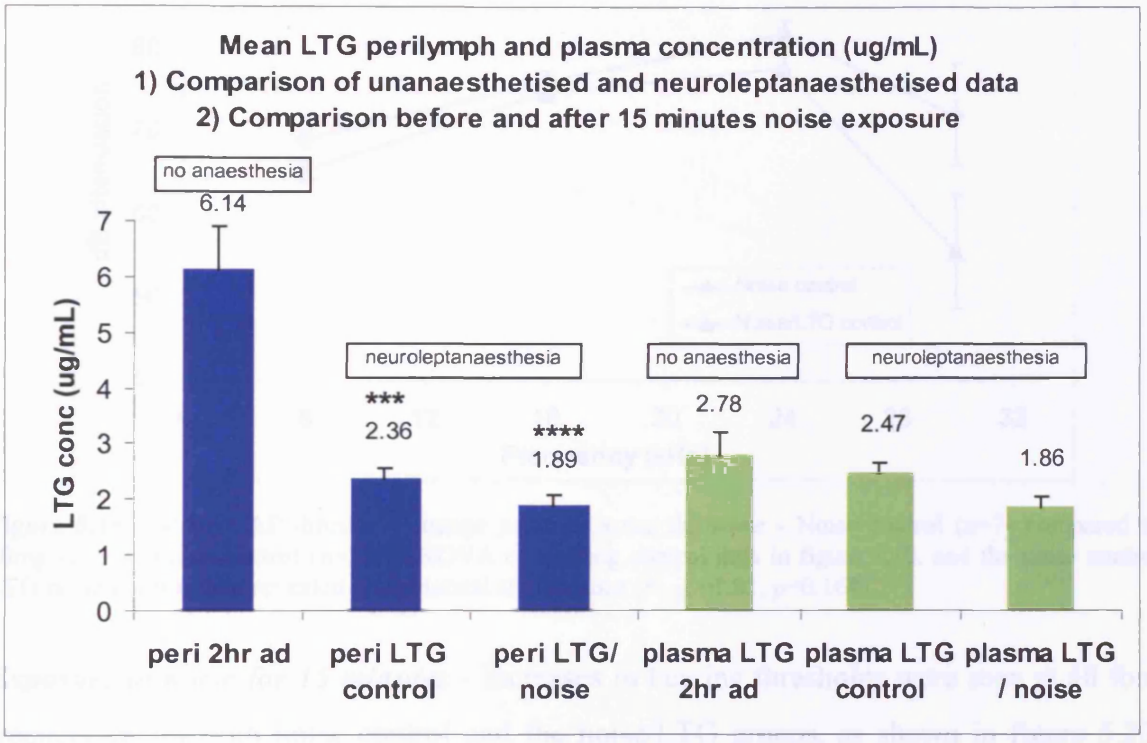


Figure 5.18. Mean LTG perilymph and plasma concentration (µg/mL) comparing *1.* No anaesthesia (n=3) against neuroleptanaesthesia data (n=5) and *2.* LTG control with 15 minute LTG/noise exposure collected 2 to 2.5 hours after dosing. No anaesthesia refers to the fact that animals were sacrificed 2 hours after LTG dosing using barbiturate overdose rather than sustained anaesthesia exposure. For perilymph results, $F_{2,9}$=46.27 p<0.0001, ANOVA. ***P<0.001, ****P<0.0001, Students t test.

5.10.6. Changes to CAP threshold following 15 minutes 100 dB SPL noise exposure – Noise vs 20mg/kg LTG/noise

Prior to noise exposure - Mean threshold values were again comparable between the noise control and noise/LTG groups with the exception of 30 kHz (figure 5.19). These results were very similar to the threshold values collected from the artificial perilymph time control group and the LTG control group as shown in figure 5.2. Again, these results were not statistically significant (t-test) and when all four treatment groups were also compared by ANOVA, again no significance was attained ($F_{3,30}$ =1.82, p=0.164).

Noise control values were 72.0±6.1, 81.9±1.5, 76.1±1.0 and 69.0±1.1, compared to 55.6±6.8, 77.6±1.9, 74.7±1.1 and 65.1±1.3 dB SPL for the noise/LTG controls for 30, 24,

16 and 8 kHz respectively. The 16 dB mean difference at 30 kHz was comparable to the 14 dB difference observed between the previous control values (see figure 5.2).

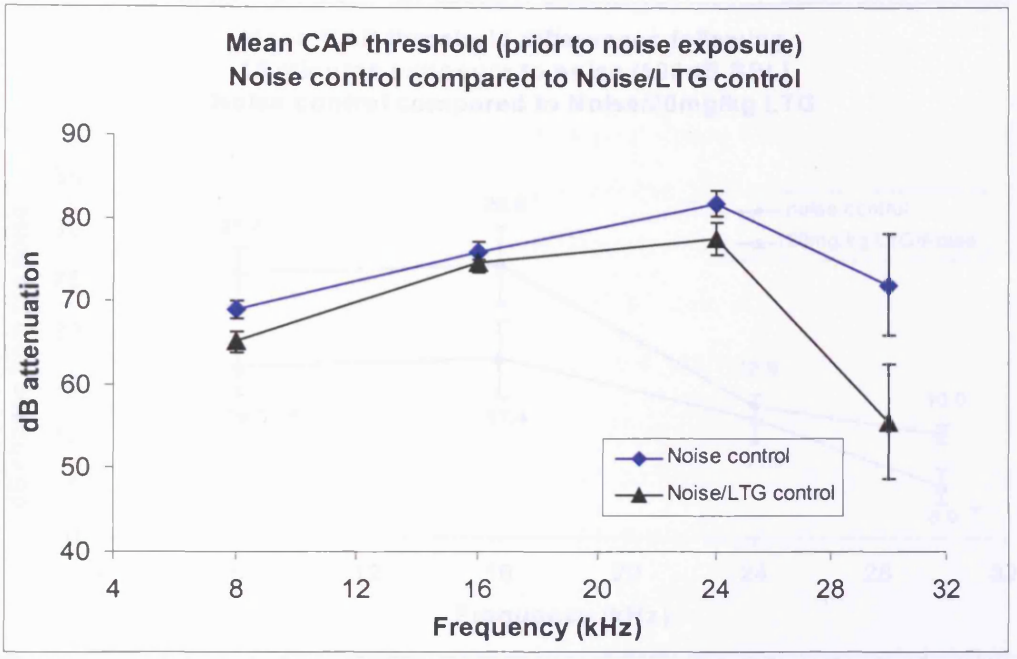


Figure 5.19. Mean CAP threshold change *prior to noise exposure* - Noise control (n=7) compared to 20mg/kg LTG/noise control (n=7). ANOVA comparing control data in figure 5.10, and the noise control LTG/noise control data revealed no statistical significance ($F_{3,30}$ =1.82, p=0.164).

Exposure to noise for 15 minutes - Increases in hearing thresholds were seen at all four frequencies for both noise control and the noise/LTG groups, as shown in figure 5.20. However, the relative mean threshold changes were not as marked in the LTG/noise group. This was especially noticeable at 8 and 16 kHz where a statistically significant lower increase in threshold was attained at 8 kHz ($p<0.05$, t-test). At 8 kHz, mean threshold changes were 25.7±2.5 and 16.6±2.7 dB SPL respectively for noise control and 20mg/kg LTG/noise (p=0.035). The threshold changes observed at 16 kHz for the LTG group were also markedly lower, but did not attain statistical significance. These threshold shifts were 26.6±3.8 and 17.4±3.8 dB SPL, noise control and 20mg/kg LTG/noise respectively (p=0.11, t-test).

The larger threshold shifts at 8 and 16 kHz seen in both groups reflects the difference in spectral intensity of the band pass filtered 100 dB SPL noise stimulus as previously described in Chapters 3 and 4. Similar threshold changes for both groups were seen at 24 kHz, with statistical significance attained at 30 kHz (p=0.624 and p=0.019 respectively). This lower elevation of threshold at 30 kHz may have been due to either an

effect of LTG during noise exposure, or an elevation of threshold seen prior to noise exposure.

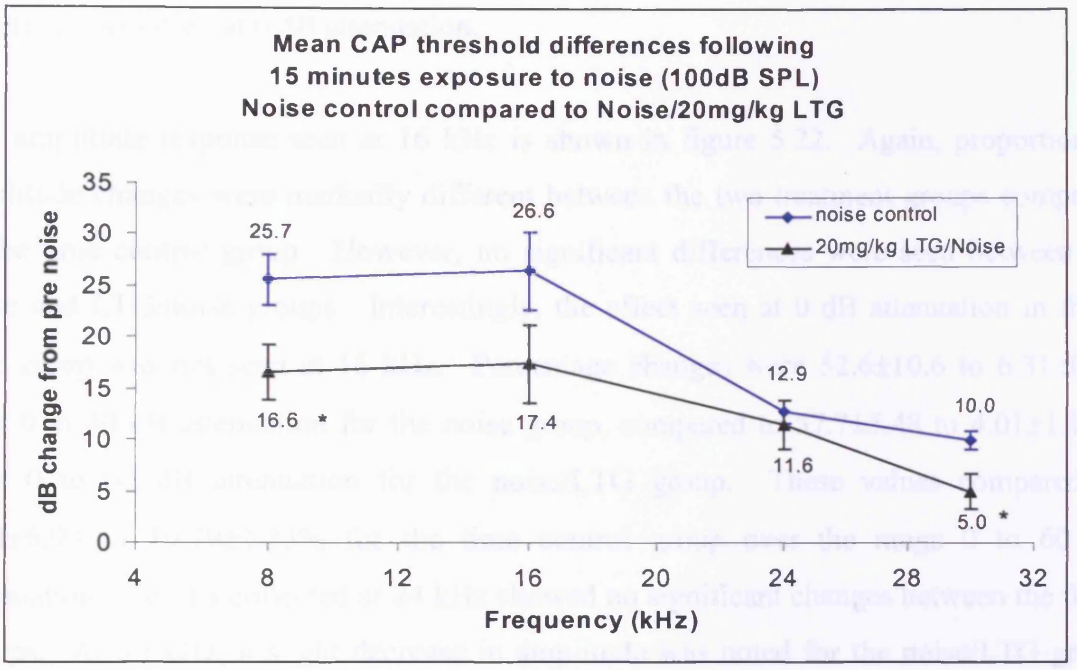


Figure 5.20. Mean CAP threshold change before during and after 15 minutes 100dB SPL noise exposure – Noise control (n=7) and 20mg/kg LTG/Noise (n=7) * = P<0.05, Students t test.

5.10.7. Changes to CAP N_1 amplitude following 15 minutes 100 dB SPL noise exposure: Noise vs 20mg/kg LTG/noise

Prior to analysis, data were normalised with respect to values at 0 dB attenuation (100%) pre noise exposure. These results were then compared with the normalised time control results in order to assess *relative* decreases in amplitude. Normalisation here was performed firstly to reduce the effects of inter animal variability. Secondly, it was done to reduce the marginal, decremental effects on amplitude seen with LTG treatment alone.

Following the 15 minute noise exposure, highly significant decreases in amplitude were noted in both the noise control and noise/LTG groups at 20 and 40 dB attenuation ($p<0.01$ to $p<0.0001$) at 8 kHz as shown in figure 5.21. *Post hoc* analysis (Tukey's test) revealed that the noise and LTG/noise groups were not significantly different from each other at these stimulus intensities. This meant that, *proportionately*, the decreases in amplitude following noise exposure in both groups were equivalent. Interestingly, the effect on proportionate amplitude change at 0 dB attenuation was different. *Post hoc* analysis (Tukey's test) showed that for the noise alone treated group, a significant proportionate increase in amplitude had occurred at this stimulus intensity when compared to the other

two groups. In percentage terms, changes compared to control over 0 to 40 dB attenuation were 113.9±9.79 to 3.1±0.84% for the noise exposed group compared to 77.6±3.24 to 8.1±2.02% for the noise/LTG group respectively, highlighting the large difference observed at 0 dB attenuation.

The amplitude response seen at 16 kHz is shown in figure 5.22. Again, proportionate amplitude changes were markedly different between the two treatment groups compared to the time control group. However, no significant differences were seen between the noise and LTG/noise groups. Interestingly, the effect seen at 0 dB attenuation in the 8 kHz group was not seen at 16 kHz. Percentage changes were 52.6±10.6 to 6.31±0.84 over 0 to 40 dB attenuation for the noise group, compared to 57.7±5.48 to 4.01±1.19% over 0 to 60 dB attenuation for the noise/LTG group. These values compared to 85.4±6.99 to 10.79±1.33% for the time control group over the range 0 to 60 dB attenuation. Results collected at 24 kHz showed no significant changes between the three groups. At 30 kHz, a slight decrease in amplitude was noted for the noise/LTG group over the range 0 to 60 dB attenuation but using ANOVA this did not attain statistical significance (figure 5.23).

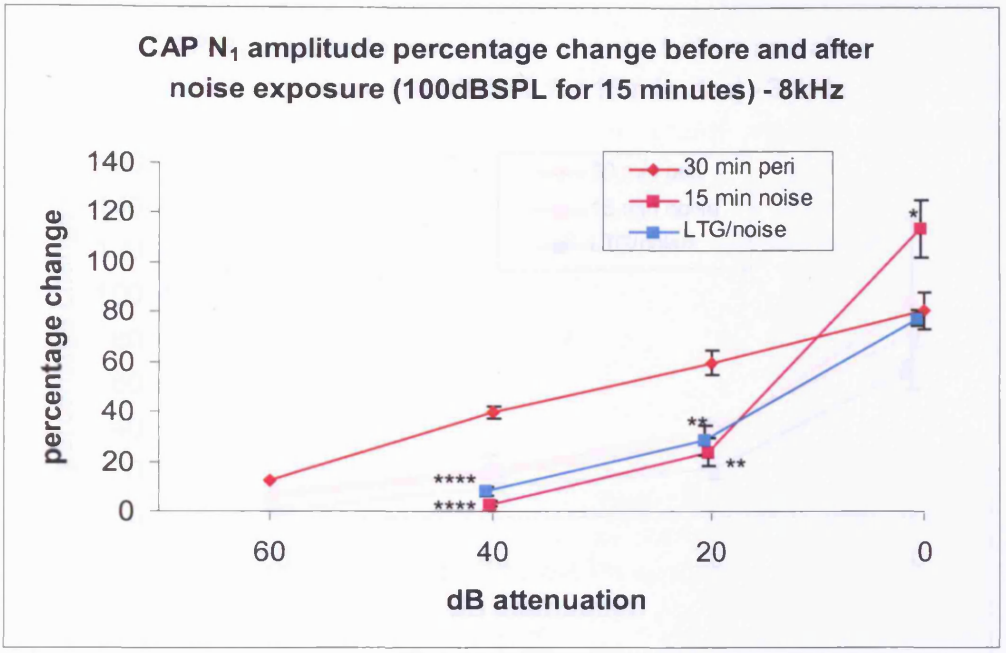


Figure 5.21. Mean CAP N_1 amplitude change at 8 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (*p<0.05, **p<0.01, ****p<0.0001, Students t-test). Data represent percentage change from pre-noise exposure compared to 0dB attenuation. ($F_{2,15}$=7.04 p<0.01, $F_{2,15}$=9.3 p<0.01 and $F_{2,14}$=32.72 p<0.0001 for 0, 20 and 40dB attenuation respectively, ANOVA).

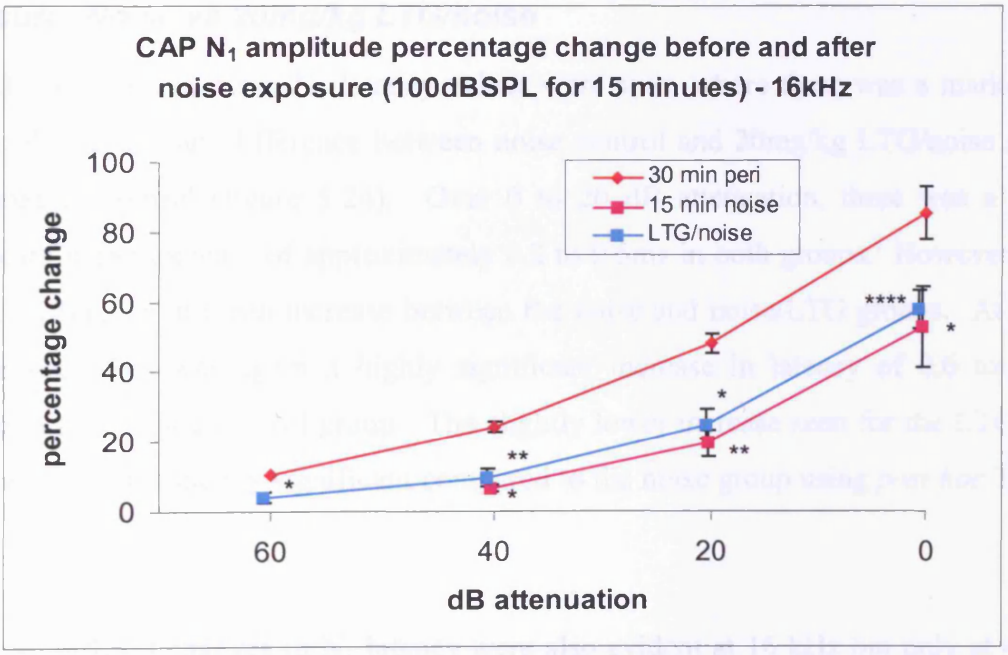


Figure 5.22. Mean CAP N_1 amplitude change at 16 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (*p<0.05, **p<0.01, ****p<0.0001, Students t-test). Data represent percentage change from pre-noise exposure compared to 0dB attenuation ($F_{2,16}$=4.38 p<0.05, $F_{2,16}$=7.77 p<0.01 and $F_{2,15}$=14.69 p<0.0001 for 0, 20 and 40dB attenuation respectively, ANOVA).

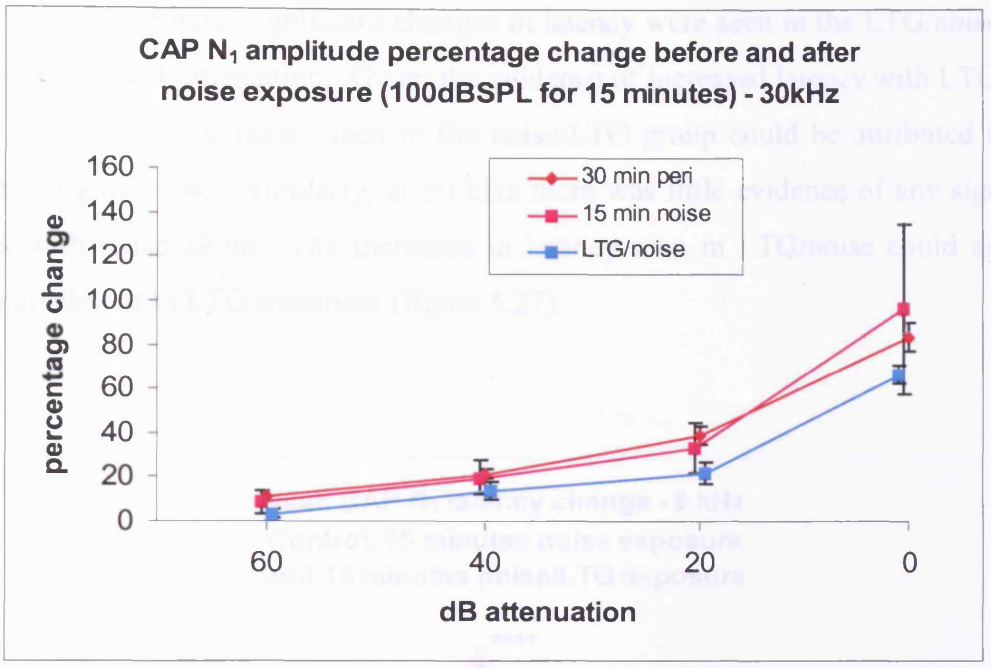


Figure 5.23. Mean CAP N_1 amplitude change at 30 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing. Data represent percentage change from pre-noise exposure compared to 0dB attenuation.

5.10.8. Changes to CAP N_1 latency following 15 minutes 100 dB SPL noise exposure: Noise vs 20mg/kg LTG/noise

At 8 kHz changes in mean N_1 latency values were seen, where there was a marked and statistically significant difference between noise control and 20mg/kg LTG/noise groups compared to control (figure 5.24). Over 0 to 20 dB attenuation, there was a highly significant mean increase of approximately 0.2 to 0.5ms in both groups. However, there was no difference in mean increase between the noise and noise/LTG groups. At 40 dB attenuation, there was again a highly significant increase in latency of 0.6 to 0.8ms compared to the time control group. The slightly lower increase seen for the LTG/noise group was not statistically significant compared to the noise group using *post hoc* Tukey's test.

Highly significant changes in N_1 latency were also evident at 16 kHz but only at 40 and 60 dB attenuation ($p<0.01$, $p<0.001$, t-test) as shown in figure 5.25. At this frequency, data obtained at 60dB attenuation were from the noise/LTG and control groups only due to treatment related threshold shifts.

At 24 kHz, significant changes in latency in the noise group were only seen at 60 dB attenuation. In contrast, significant changes in latency were seen in the LTG/noise group at 20, 40 and 60 dB attenuation. Given the evidence of increased latency with LTG alone, at least some of the increases seen in the noise/LTG group could be attributed to LTG treatment (figure 5.26). Similarly, at 30 kHz there was little evidence of any significant changes with noise alone. The increases in latency seen in LTG/noise could again, in part, be attributed to LTG treatment (figure 5.27).

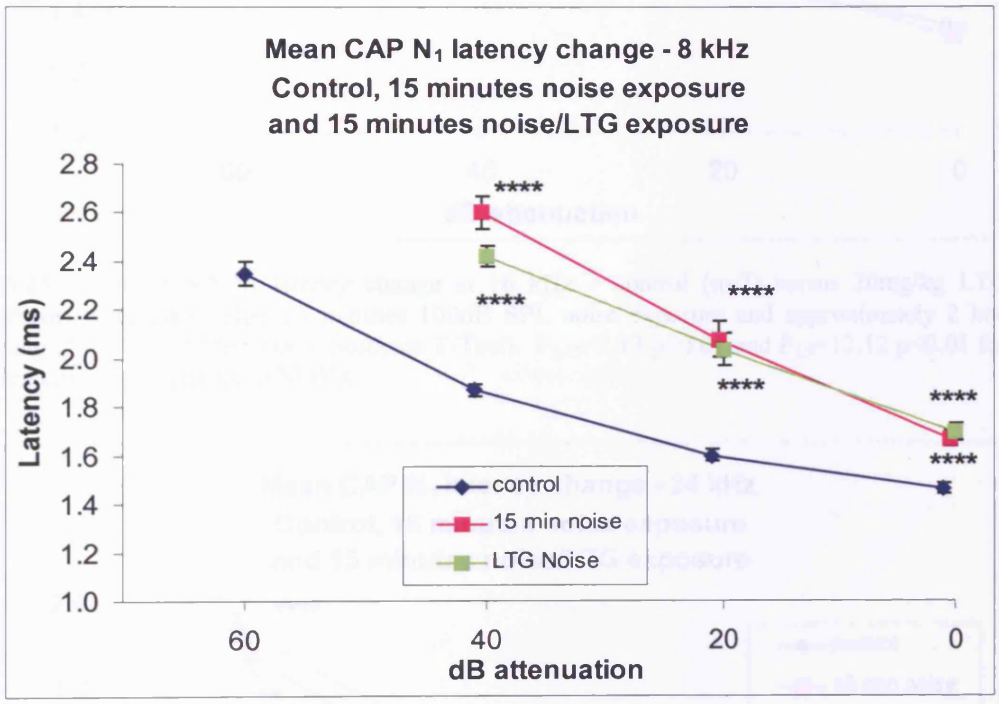


Figure 5.24. Mean CAP N_1 latency change at 8 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (****$p<0.0001$, Students t-test). $F_{2,18}=16.06$ $p<0.0001$, $F_{2,17}=17.16$ $p<0.0001$ and $F_{2,14}=57.04$ $p<0.0001$ for 0, 20 and 40dB attenuation respectively, ANOVA.

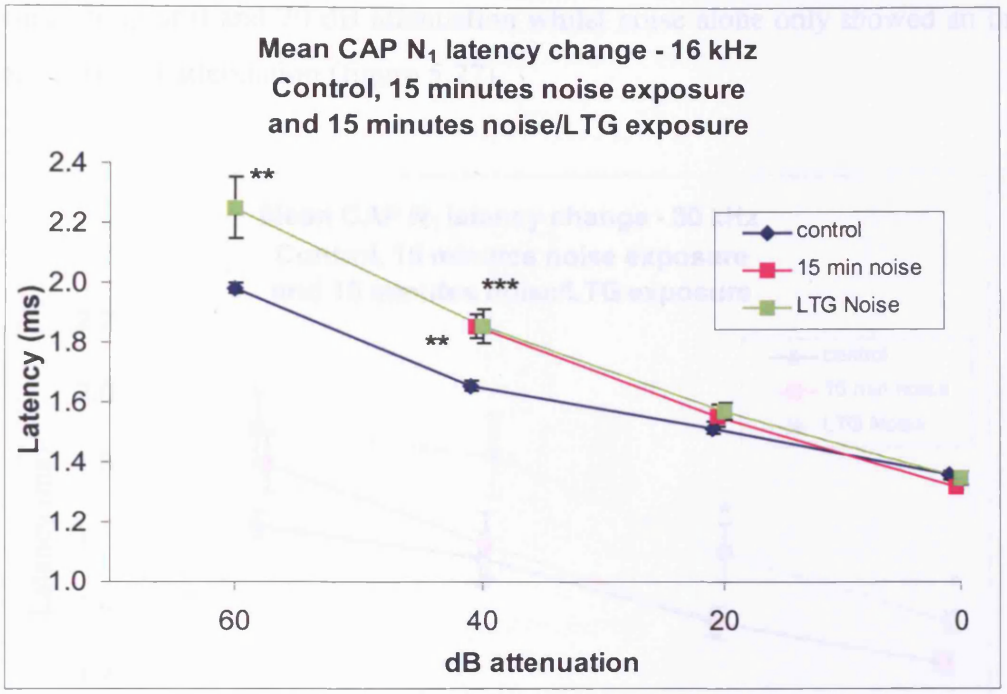


Figure 5.25. Mean CAP N_1 latency change at 16 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (**p<0.01, ***p<0.001, Students T-Test). $F_{2,17}$=7.13 p<0.01 and $F_{1,9}$=12.12 p<0.01 for 40 and 60dB attenuation respectively, ANOVA.

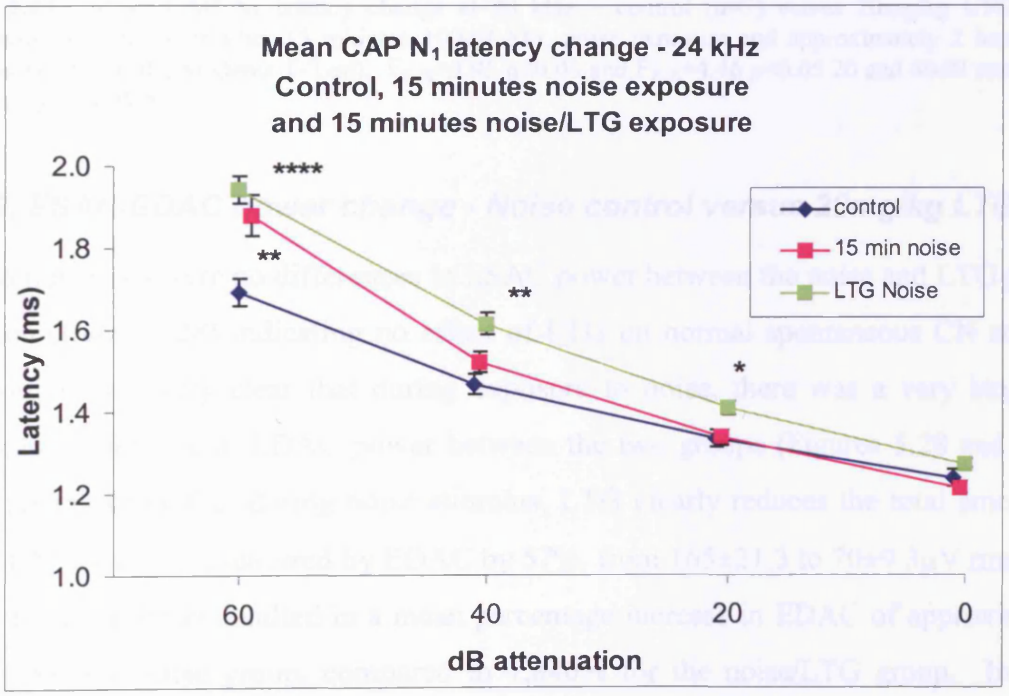


Figure 5.26. Mean CAP N_1 latency change at 24 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (* p<0.05, **p<0.01, ****p<0.0001, Students t-test). $F_{2,18}$=5.45 p<0.05, $F_{2,18}$=8.87 p<0.01 and $F_{2,16}$=10.02 p<0.001 for 20, 40 and 60dB attenuation respectively, ANOVA.

Similarly, at 30 kHz statistical differences compared to control were observed in the LTG/noise group at 0 and 20 dB attenuation whilst noise alone only showed an increase in latency at 60 dB attenuation (figure 5.27).

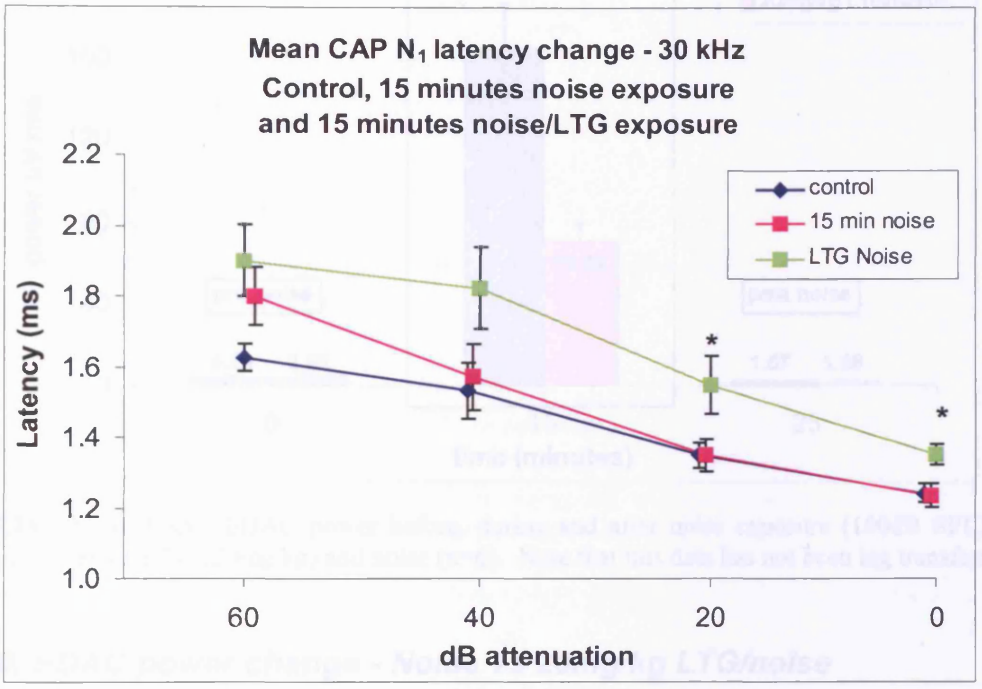


Figure 5.27. Mean CAP N_1 latency change at 30 kHz – control (n=7) versus 20mg/kg LTG (n=7). Measurements were taken after 15 minutes 100dB SPL noise exposure and approximately 2 hours after LTG dosing (* $p<0.05$, Students T-Test). $F_{2,18}=3.95$ $p<0.05$ and $F_{2,18}=4.46$ $p<0.05$ 20 and 40dB attenuation respectively, ANOVA.

5.10.9. ESAC/EDAC power change - Noise control versus 20mg/kg LTG

Prior to noise exposure no differences in ESAC power between the noise and LTG groups was seen (figure 5.28) indicating no effect of LTG on normal spontaneous CN activity. However, it was very clear that during exposure to noise, there was a very large and significant difference in EDAC power between the two groups (Figures 5.28 and 5.29). These results show that during noise stimulus, LTG clearly reduces the total amount of driven CN activity as measured by EDAC by 57%, from 165±21.3 to 70±9.3µV rms. The values for each group resulted in a mean percentage increase in EDAC of approximately 3,930% for the noise group, compared to 1,840% for the noise/LTG group. In other words, a highly significant 18 to 39 fold increase over basal metabolic demand.

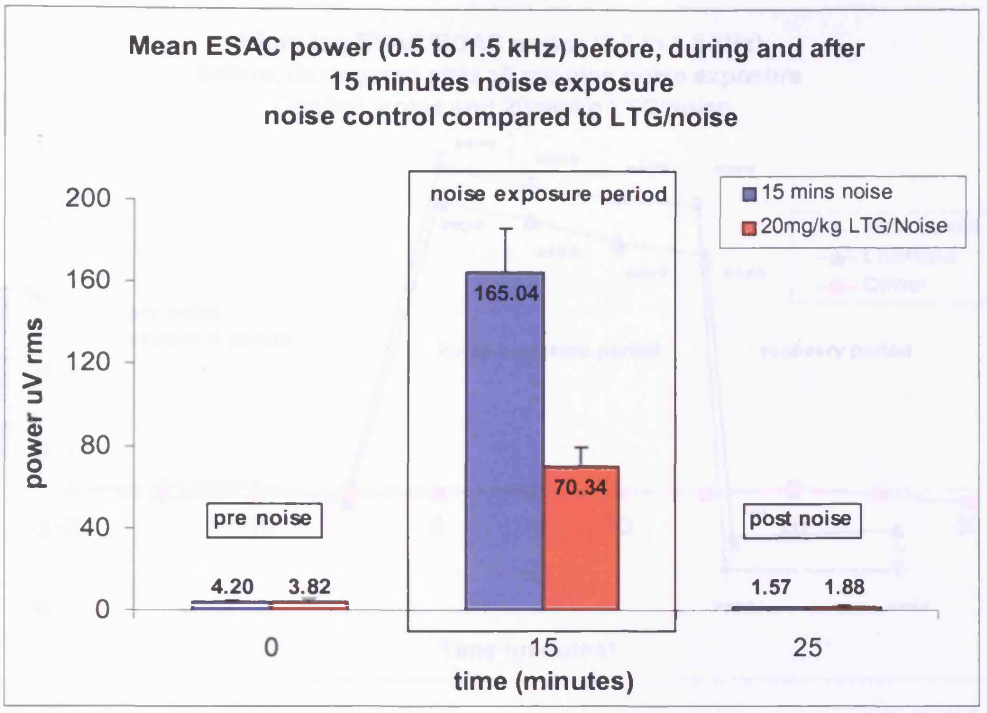


Figure 5.28. Mean ESAC/EDAC power before, during and after noise exposure (100dB SPL). Noise control (n=7) versus LTG (20mg/kg) and noise (n=6). Note that this data has not been log transformed.

5.10.10. EDAC power change - Noise vs 20mg/kg LTG/noise

Results were log transformed and all changes expressed in dB power change. Compared to control, a highly statistically significant reduction in EDAC log power was seen in both the noise and the noise/LTG treated group (figure 5.29). Mean changes in EDAC log power over the 15 minute noise exposure period ranged from 16.8±0.7 to 14.7±0.5 for the noise group compared to 14.9±0.4 to 12.2±0.7 dB for the noise/LTG group. At each point during noise exposure, the increase in driven CN activity was significantly lower for the LTG/noise group compared to the noise group.

Following noise exposure, reductions in ESAC log power were seen in both the noise and the LTG/noise groups compared to control, but the decrease in power was not as marked for the noise/LTG group as for the noise group. The values, as shown in figure 5.29, were -2.4±0.6 to –2±1.1 dB for the LTG/noise group, compared to -4.1±0.4 to -4.1±0.4 dB for the noise group.

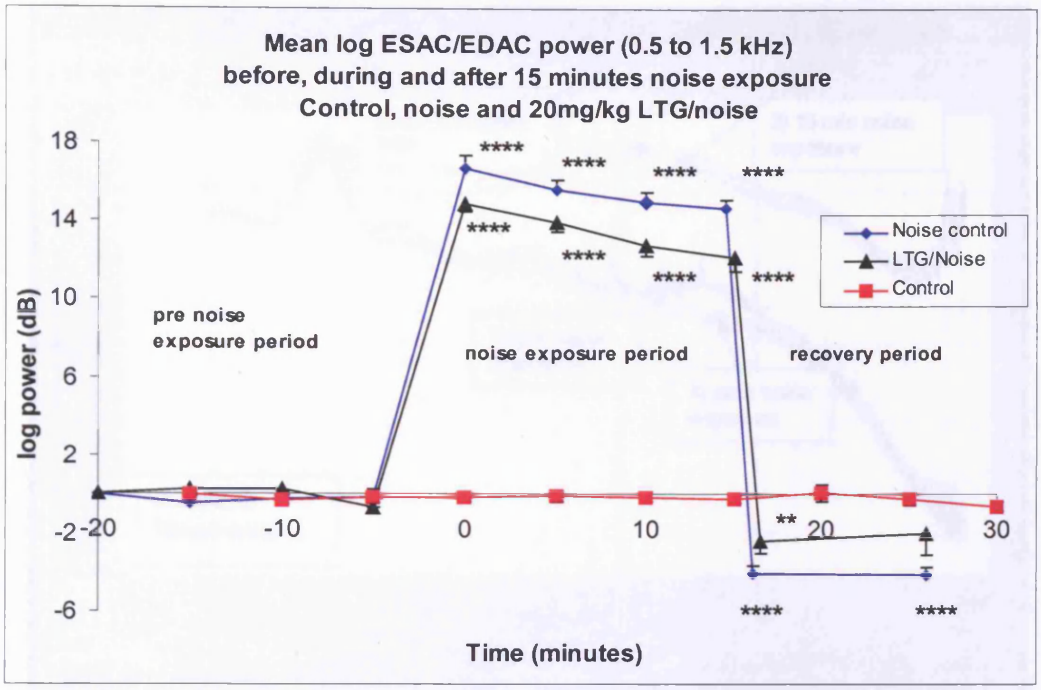


Figure 5.29. Mean log ESAC/EDAC power before, during and after noise exposure (100dB SPL). Noise control (n=7) LTG (20mg/kg) and noise (n=6) versus control (n=6). **p<0.01, ****p<0.0001 (Student's t-test). $F_{2,16}$=327.44, $F_{2,16}$=439.73, $F_{2,14}$=202.02 and $F_{2,16}$=222.41 for 0 to 15 minutes noise exposure respectively, and $F_{2,15}$=20.95 and $F_{2,10}$=22.05 for post noise exposure (all p<0.0001, ANOVA).

5.10.11. Raw ESAC/EDAC data - Noise vs 20mg/kg LTG/noise

Figures 5.30 and 5.31 show raw ESAC/EDAC screen printouts taken from representative animals in the noise control and LTG/noise groups. During noise exposure, a distinct pattern of change was observed between the two groups, notably between 0.5 and 1.5 kHz. The most noticeable difference was the appearance of a marked power 'hump' for the LTG/noise group (figure 5.31) that was absent in the noise control animals (figure 5.30). This hump was characterised by two features; a distinct 'shoulder' at approximately 1.3 kHz, and a downward shift in the power peak of the hump from approximately 1 to 0.75 kHz. This change was representative of other animals in the LTG/noise group although not all within this group showed this change.

These changes in the noise/LTG EDAC are suggestive of at least two separate processes in the opening and closing of Na^+ ion channels. On the FFT plot shown in figure 5.31, the clear 'shoulder' around 1.3 kHz provides evidence for these two components. The 'slower' of these two components in this example exhibits a reduction in the rate of its' kinetics, shifting from 1 kHz down to 0.75 kHz. As well as the power shape change around 1 kHz, the slope gradient up to 8 kHz appeared to be shallower than the control. These, however, were not analysed in detail.

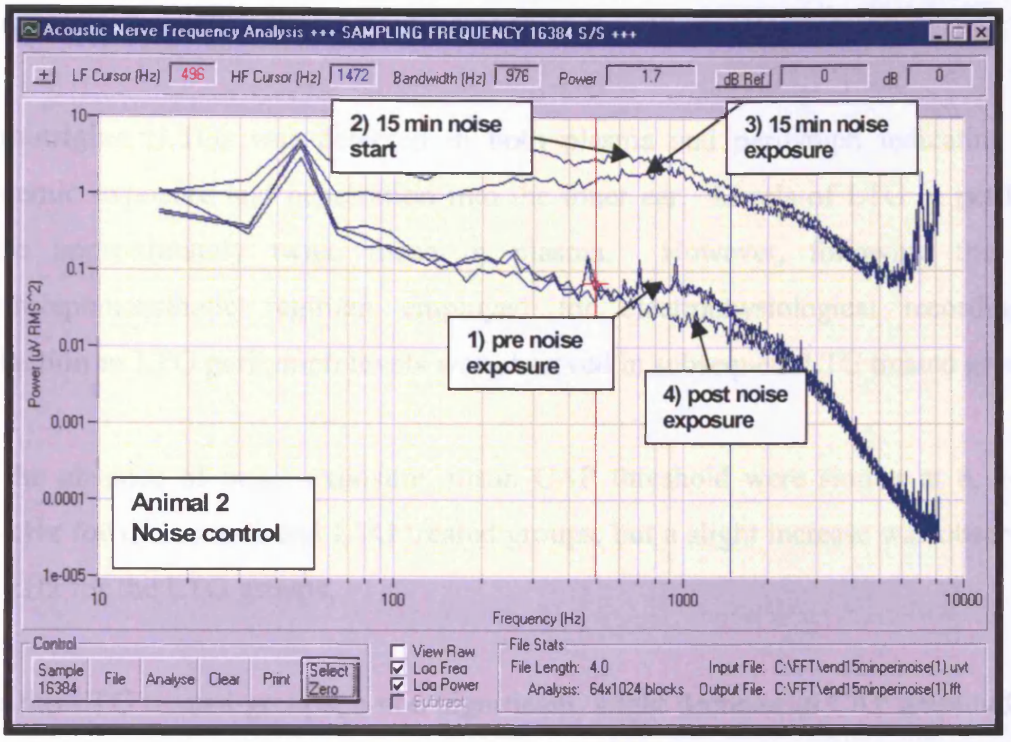


Figure 5.30. Raw ESAC/EDAC printout capturing recordings 1) pre noise exposure 2) the start of 15 minutes noise exposure 3) last recording taken prior to the end of the 15 minute noise exposure and 4) post noise exposure. Note the large increase in power during the noise exposure period and the drop in power around 0.6 to 1.5 kHz post noise exposure. The marked change in power slope observed after 8 kHz reflects interference generated by the 100 dB SPL noise stimulus.

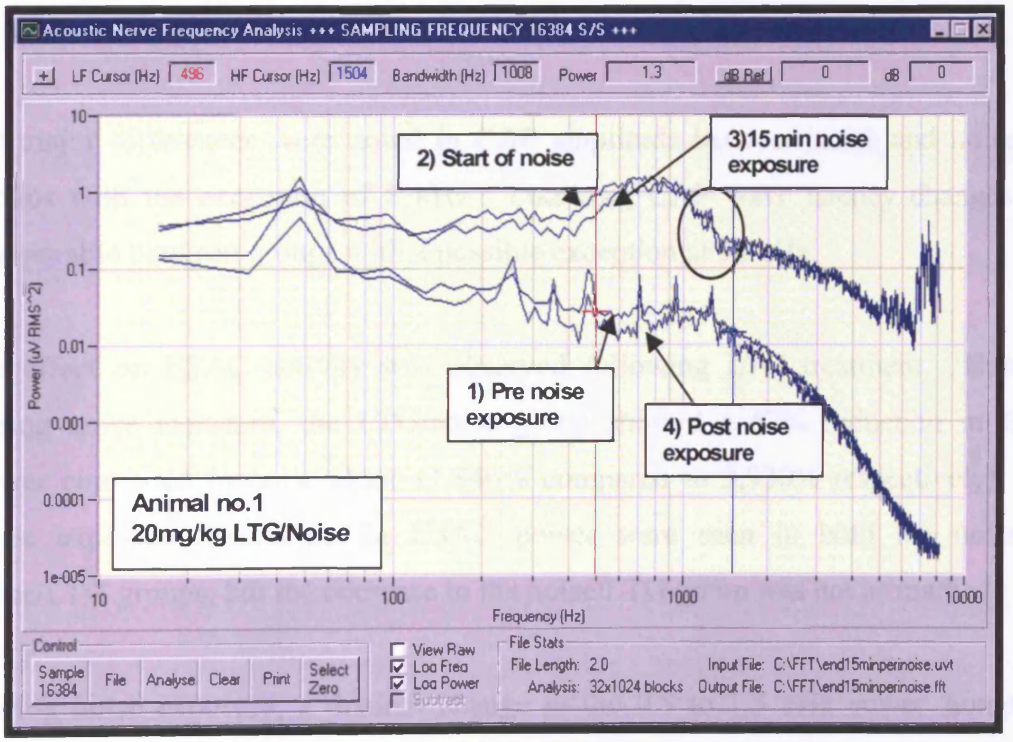


Figure 5.31. Raw ESAC/EDAC printout capturing recordings 1) pre noise exposure 2) the start of 15 minutes noise exposure 3) last recording taken prior to the end of the 15 minute noise exposure and 4) post noise exposure. All recordings were taken between 2 and 2.5 hours after LTG dosing. Note again the large increase in ESAC power during noise stimulus but a marked difference in the power spectrum shape around 500 to 1500 Hz. Compared to noise exposure alone, there was as a sharp 'shoulder' indicated by the circle.

To summarise, the results from Chapter 5 showed the following:

- Lamotrigine (LTG) was detected in both plasma and perilymph indicating good systemic exposure and penetration into the inner ear. Levels of LTG in perilymph were approximately twice those in plasma. However, following the usual neuroleptanaesthetic regimen employed for electrophysiological recordings, a reduction in LTG perilymph levels was observed in subsequent LTG treated groups.

- In the absence of noise exposure, mean CAP threshold were similar at 8, 16 and 24 kHz for the control and LTG treated groups, but a slight increase was observed at 30 kHz for the LTG groups.

- For the LTG treated groups, a non-significant, slight decrease in CAP amplitude was observed at all frequencies tested, which was slightly more noticeable at 30 kHz. No clear tonotopic distinction in CAP latency changes was observed.

- Mean CAP threshold increases following noise exposure were not as marked in the LTG/noise group especially at 8 and 16 kHz.

- No major differences were noted in CAP amplitude between noise and noise/LTG groups with the exception of 8 kHz. Likewise, CAP wave latency changes were comparable between groups with a possible exception at 30 kHz.

- No effect on ESAC activity was observed following LTG treatment. However, during noise exposure, the LTG/noise group showed a 57% reduction in EDAC power compared to noise alone (1,840% compared to 3,930% respectively). Post noise exposure, reductions in ESAC power were seen in both the noise and noise/LTG groups, but the decrease in the noise/LTG group was not as marked.

- During noise exposure, a distinct change in the 0.5 to 1.5 kHz power 'hump' was observed following treatment with LTG shown as a distinct 'shoulder' at approximately 1.3 kHz.

Consideration of these effects will now be discussed in section 5.11.

5.11. Discussion and conclusions

5.11.1. Pharmacokinetics (PK) of LTG before during and after noise exposure

Lamotrigine (LTG) was detected in both plasma and perilymph indicating good systemic exposure and penetration into the inner ear (Figure 5.8). These results show the preferential uptake of LTG by the target organ, the cochlea. These results were not completely unexpected as the tissue:plasma ratio for LTG throughout the body is usually greater than 1. This is most noticeable in heavily pigmented tissues such as the liver and kidney (Parsons *et al*, 1995). The brain:plasma ratio is typically between 1.6 to 2 (Parsons *et al*, 1995) and the peak perilymph:plasma ratio values of 2.2 in this chapter falls within this range suggesting that the PK of LTG in the cochlea would be similar to that of the brain (Parsons *et al*, 1995).

The half-life of LTG in both plasma and perilymph also appeared to be comparable at about 12 hours providing significant exposure. From the point of view of using LTG as a putative cochleoprotectant this was encouraging as it suggested that adequate plasma/perilymph levels could be maintained with either a once or twice daily dosing regime.

Published plasma C_{max} values after a clinical dose of LTG (3.1μg/mL, Table 5.1) are very similar to the values for guinea pig plasma LTG obtained in this chapter of 3.6±0.8μg/mL. This suggests that the choice of 20mg/kg LTG used in this study was a valid dose in establishing an animal model for protection against NIHL. This also had direct clinical relevance, as it yielded plasma concentrations that were well within the anticonvulsant therapeutic range of 1-10μg/ml or 4-40μM (Leach *et al*, 1995).

The results gained during electrophysiological experimentation under neuroleptanaesthesia were unexpected (figure 5.18). These showed that perilymph LTG concentrations were at least two-fold lower than those collected during the initial control LTG PK experiments gained without neuroleptanaesthesia. This is of interest from both a PK and a cochleoprotectant point of view. From the pharmacokinetic viewpoint this is relevant because, if these results were a true reflection of the perilymph levels, it suggests that the difference was a real treatment effect. This effect also appeared to be specific to the cochlea, as plasma levels were unaffected. From the cochleoprotectant viewpoint, it

means that significant protection could be afforded at relatively lower perilymph concentrations, ie about 7.5-10μM compared to about 25μM in the unanaesthetised preparation.

If these results are correct then this finding has considerable implications for how LTG, and presumably other VDSCMs, are distributed throughout the body. It would therefore be of value to reassess the pharmacokinetics of LTG under neuroleptanaesthesia, in a number of other compartments as well as the cochlea and plasma.

Whilst a definitive explanation for these results is not forthcoming, various plausible explanations are presented below:

The perilymph sampling procedure may have introduced contamination between plasma and perilymph - This is very unlikely given the very successful sampling shown in the PK profile study resulting in low standard error values. Also, it is unlikely that all animals would have been incorrectly sampled.

An error in the dose of LTG administered - This is very unlikely due to the fact that the same very simple formulation protocol was followed for both control LTG and noise exposed LTG animals. Any error in dosing would have been reflected in a high degree of inter animal variability as each formulation was made fresh prior to each experiment. In addition, plasma LTG levels did not significantly change indicating very similar exposure.

Error during sample analysis - During sampling analysis, an error in aliquot sampling may have occurred due to the very low volumes collected resulting in apparently lower perilymph LTG values. As a dilution factor was not applied to the plasma samples this is certainly a possible explanation. However, retrospective inspection of data collected in laboratory notebooks did not indicate any such error.

Increased metabolism of LTG due the effects of anaesthesia during surgery - Yuen *et al* (1992) and Remmel and Sinz (1991) state that LTG is mainly metabolised by hepatic glucuronidation, and drugs that are metabolised via this route may potentially increase the rate of LTG clearance. A report in the rat states that the metabolites of medetomidine comprise a metabolic pathway of hydroxylation with subsequent glucuronidation, or

further oxidation to carboxylic acid (Salonen and Eloranta, 1990). However, no strong evidence exists to suggest that the anaesthetics used in this thesis (either medetomidine, fentanyl or Isoflurane) affected the pharmacokinetic profile of LTG especially as plasma LTG levels appeared to be unaffected. Moreover any effect would have to be specific to the cochlea alone.

5.11.2. Effects on CAP threshold – LTG control

A single oral dose of 20mg/kg LTG did not appear to significantly alter CAP threshold data, when measured approximately 2 hours after dosing. This was apparent in both sets of experiments where the LTG alone group and the noise control and LTG/noise groups were compared. Whilst there was no evidence of significant threshold increase at 30 kHz, in both sets of these experiments, thresholds were elevated by about 12 –14 dB ($p \sim 0.13$ in both cases). This contrasted with the very close mean thresholds obtained for all four treatment groups over 8 to 24 kHz. Over these frequencies, LTG would appear to be having no effect at all on CN fibre sensitivity.

In explaining this relatively marked, though non-significant increase in threshold, three possibilities are proposed here. The first one is that this elevation simply reflected the greater sensitivity of the basal (ie. high frequency) part of the cochlea to general insult eg. surgical procedure that may simply have occurred in the two groups of LTG treated animals.

The second proposal is that the resting membrane potential of higher frequency fibres may be at a lower value than in the more apical, lower frequency regions of the cochlea. If this was the case, then the lower resting potential would result in a greater proportion of Na^+ channels existing in the inactivated state, allowing LTG more access to its binding site (Xie *et al*, 1995, and Xie and Garthwaite, 1996). This is described in more detail in section 5.4.3.

The third proposal relates to HVA Ca^{2+} channels. Because VGCCs are responsible for the repolarisation phase of the action potential, they can strongly influence action potential propagation. In addition to this, VGCCs control synaptic release in the vast majority of central pathways in particular P, Q and N-type channels. This governs Ca^{2+} entry at presynaptic nerve terminals, regulating both excitatory and inhibitory post synaptic currents (Stefani *et al*, 1997). Based on the work by Wang *et al* (1996) Stefani *et al*

(1996) Von Wegerer *et al* (1997) and Hainsworth *et al* (2001) there is evidence to suggest an action of LTG on HVA Ca^{2+} channels, albeit a modest one. These data, together with the presence of HVA L, P/Q and R-type channels in the cochlea (Ertel *et al*, 2000) provide a solid basis for further investigation.

5.11.3. Effects on CAP N_1 amplitude – LTG control

Over all frequencies and suprathreshold stimulus intensities, there were consistent though non-significant decreases in amplitude in the LTG alone group compared with the time control group. These decreases were most marked at 30 kHz where they fell by as much as 75%. A comparable trend in reduced amplitude, pre-noise exposure, was also seen in the LTG/noise group when compared with the noise control group. This also included a more marked suppression of amplitude at 30 kHz. This suggests that this reduction in amplitude, whilst not significant, may have reflected a weak but real tonotopic effect of LTG on the CN. As stated in the results section, strict comparison with the time control group was not completely justified. This was because there was no pre-LTG treatment data, and only one time point at which data was measured at 2 hours post dosing. This compromise was necessary to allow perilymph sampling at this time point in this experimental group. Notwithstanding this limitation, a number of possible reasons for these effects on amplitude are briefly considered.

Firstly, a systemic effect of LTG, such as reducing systemic blood pressure, was not likely, as cochlear thresholds would also have been expected to be affected (Evans, 1972, Mulheran and Evans, 1986, Mulheran 1990).

Secondly, an effect of LTG on the discharge frequency of suprathreshold spike generation may be possible. Normally LTG, at least in other experimental systems using the same concentration as in this study, was not thought to affect the normal physiological response of the neurone (Lees and Leach, 1993). However, this and other previously referenced studies appear to have been carried out in models with lower driven activity. In the figures from these references they appear to be between 10-100 spikes/sec, at least *in vitro*. As mentioned previously, guinea pig cochlear afferent fibres have driven activities that go well above ie up to 450 spikes/sec (Cooper, 1989 and Mulheran, 1990) as shown in figure 5.32. This rate of driven activity could lead to a greater 'post spiking' level of depolarisation that would then be expected to result in a probabilistically greater number of channels in the inactivated state (Xie *et al* 1995, Xie and Garthwaite, 1996). The

resultant outcome would be proportionately more LTG gaining access to its Na^+ channel-binding site. Again, as described in section 5.11.2, involvement of HVA Ca^{2+} channels cannot be completely discounted.

It is possible that LTG may have led to a reduction in individual spike amplitude, though the literature does not support this. LTG is only reported to reduce the *probability* of spike discharge in depolarised neurones, by increasing the duration of the inactivation period (Lees and Leach, 1993, Xie *et al*, 1995). This reduced discharge probability would then explain the trend in reduced amplitude. The more marked effect on amplitude seen at 30 kHz may also be due to incorporation of the previous effects seen on threshold.

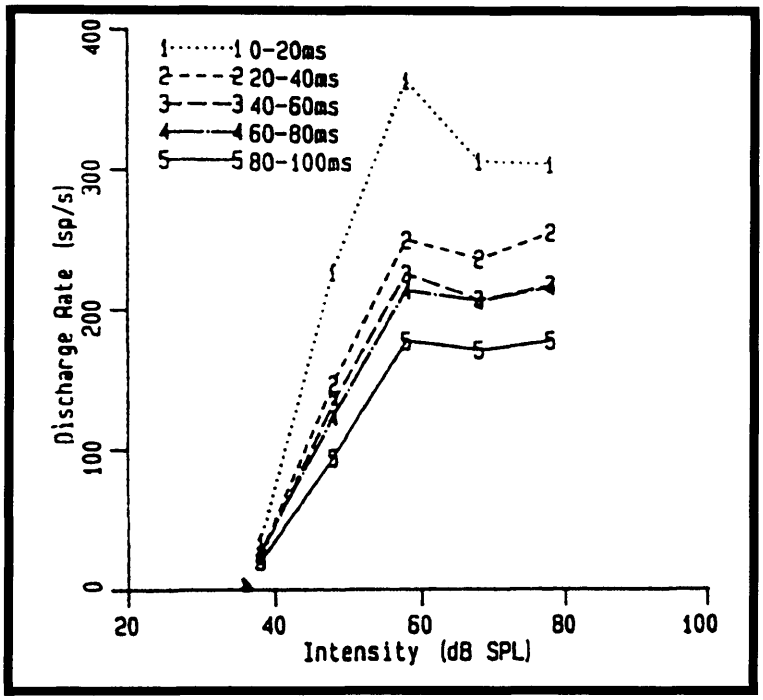


Figure 5.32. The mean activity (discharge rate) of a CN fibre measured at approximately 8 kHz. Data are based on approximately 4000 spikes recorded at each stimulus. Note the very high discharge rate within the first 20ms of stimulus (as indicated by number 1 on the plot) and then a stabilisation of activity over time. Adapted from Cooper (1989).

5.11.4. Effects on CAP N_1 latency – LTG control

No significant effects on latency were seen at 8, 24 and 30 kHz but, curiously, significant effects were seen at 16 kHz. The previous caveat about comparison of LTG alone with time controls would apply less strongly here as latency is a more absolute measure of temporal ion channel activity, which shows much less inherent variability than amplitude. This tonototopic dependence on concentration would perhaps explain the trend seen in threshold and amplitude at 30 kHz, but would not explain the increase in mean latency

seen here of 0.05 to 0.1ms. LTG *in vitro* has been shown to increase the duration of the Na^+ channel slow inactivation (figure 5.1) affecting probability of time to next activation and opening at concentrations of around 50μM (Xie *et al*, 1995). It is possible that this effect may have contributed to the significant but marginal increase in latency seen at 16 kHz, but uncertainty exists as to why a significant effect should be seen at this frequency and not others.

5.11.5. Effects on CAP threshold - LTG and noise exposure

After noise exposure, the noise control group showed significant changes in threshold over 8 to 24 kHz, with substantial increases of about 25 dB at 8 to 16 kHz. At these two frequencies LTG ameliorated the threshold shift by approximately 10 dB. This was significant at 8 kHz but at 16 kHz was non-significant ($p=0.11$). This was, however, still considered to be physiologically and pharmacologically noteworthy. The effects seen at 24 and 30 kHz were less marked due to the band pass filtering on the noise insult.

These results represents the first report of evidence that LTG can protect against acute noise damage. This is a very important finding in the development and identification of safe compounds for use as putative cochlear protectants, a fact which has been recognised by GSK who have used these findings to file application for patent on VDSCMs (WO 02/28394 A1 - see Appendix 2 for full details).

What is also important is that the results provide evidence that noise induces substantial functional deficit within the CN itself. This is based on the assumption that LTG does not have a substantial protective effect at other sites within the cochlea. This damage may be subsequent to, but is *distinct* from damage within the cochlea, and excitotoxicity restricted to an effect at the site of the afferent post synaptic process. The mechanism underlying this damage is very likely to follow the steps outlined by Taylor and Meldrum (1995) in section 5.6. Essentially this involves excessive entry of Na^+ ions into the axon that overwhelms the homeostatic response. In this case the level of Na^+ entry would not be considered to be sufficient to lead to cell death, but would lead to acute deficit in normal signal transmission by the neurone. In this case LTG blocks excessive Na^+ entry allowing the neurone to recover more rapidly.

This dissection of cochlear damage sites by treatment with LTG has shown that with the noise stimulus used in this study, on average at least 10 dB of threshold elevation is due to

nerve dysfunction in the axonal portion of the nerve fibre. The remaining 15 dB or so of threshold elevation is due to effects at sites elsewhere in the cochlea. This again has not been reported previously and represents an important advance in our understanding of cochlear response to noise.

5.11.6. Effects on CAP N_1 amplitude – LTG/Noise

It was clear that noise had a highly significant effect on amplitude at 8 and 16 kHz. This was true for both the noise control and LTG/noise groups compared to the time control group. Interestingly, at 8 kHz there was no difference between noise control and LTG/noise groups between 20 and 40dB attenuation. There was however, a significant finding in CAP N_1 amplitude at 0dB attenuation (figure 5.21). Here it was noticeable that the noise control group had a significantly greater amplitude value than either the time control or noise/LTG groups. The reason for the greater change in the amplitude at 0dB attenuation is explainable in terms of damage to the OHCs (see chapter 4, section 4.14.2.2). Here the noise affects OHC function in such a way that it partially unmasks the passive growth function of the BM. At these sound intensity levels the attenuating properties of the OHCs have been damaged. This is then reflected in the steeper input-output function seen in figure 5.21. In contrast, the input-output for the LTG/noise group was less steep, and its proportionate amplitude was comparable to the time control group.

This can be explained in terms of LTG suppressing the greater afferent neuronal discharge rate that would normally accompany passive mechanical recruitment in amplitude. Supporting evidence for this comes from the effects already seen on amplitude in the LTG alone group (figures 5.10 to 5.13). At 16 kHz, N_1 amplitude in both treatment groups was highly significantly affected compared to the time control group. However, the noise damage here would not appear to have been as severe as at 8 kHz with the input-output function of noise/LTG being directly comparable with the noise group. Proportionately, amplitude did not significantly change at either 24 or 30 kHz, reflecting the fact that the noise stimuli experienced by these locations within the cochlea was essentially sub traumatic.

5.11.7. Effects on CAP N_1 latency – LTG/Noise

At 8 kHz there was a highly significant effect on latency. However, there was no difference between the increases in latency in either treatment groups. This was also true for 16 kHz but was less marked. Curiously, at this frequency, these results contrast those

found with LTG alone (figure 5.15) suggesting that the effect of LTG alone on latency may not be consistent.

This inconsistency in latency changes was also seen at 24 and 30 kHz, where noise alone at most intensities did not appear to have any significant effect. In the noise/LTG group there was a slight increase in latency (0.05 to 0.1ms) over the noise alone group. The reasons for this are unclear but may possibly represent a post noise-induced decrease in resting membrane potential at these frequency loci. To establish whether this was a real effect would require repeat experimentation.

5.11.8. Effects on ESAC and EDAC - LTG control and LTG/noise

Further interesting and novel results were generated from the ESAC/EDAC studies. Figures 5.28 and 5.29 showed that LTG alone did not affect ESAC activity, providing evidence that normal spontaneous CN activity remained unaffected. These experiments also showed that during noise exposure, significant increases in the driven CN activity amounted to approximately 3,900%. This provides an indirect measure of the increase in metabolic demand required by the nerve in response to these stimuli and would amount to a probable near 40-fold increase in requirement for ATP. In addition, this provides a novel insight into the stress the CN has to contend with when subject to this type of stimuli in 'real life'. This would also be expected to place both the neuron and proximal glial cells under considerable stress. After LTG treatment, EDAC power was dramatically reduced to approximately 1,800% representing an overall change in power of about 58%. This fits in with the scheme proposed by Taylor and Meldrum (1995) but this study here provides quantitative evidence of a probable reduction in metabolic demand.

Underlying the decrease in EDAC would be the reduced probability of neuronal discharge in excessively stimulated nerve fibres. These EDAC results also support the idea proposed previously that LTG is acting to reduce the amplitude of driven activity in the CN (figures 5.21 to 5.23)

The outcome of the reduction in EDAC activity is that the potential for CN deficit is reduced, as shown post noise stimulus where the reduction in EDAC offered by LTG resulted in improved post noise ESAC activity compared to noise control data.

The reason for this decrease in post noise ESAC activity is likely to be the result of a number of mechanisms including excitotoxicity and recovery of the nerve itself from increased metabolic demand. The ameliorating effect of LTG on this deficit is again further evidence that the CN axonal process is an independent site of damage following noise exposure. Histopathology studies are not presented in this thesis, therefore supporting structural evidence of cochlear damage cannot be provided.

5.11.8.1. Effects of LTG on the appearance of the EDAC power spectrum

Figure 5.31 shows the effect of LTG on the appearance of the EDAC power spectrum in the region of 0.5 to 1.5 kHz where a distinctive change in appearance of the 'power hump' was observed in 3 out of the 7 animals. This distinctive hump strongly suggests, at least in these animals, that LTG was changing Na^+ channel kinetics and possibly Ca^{2+} channels also.

This frequency component of the FFT spectrum would match the activation and fast inactivation components of the VGSCs. This shift in the spectrum could therefore be taken as evidence that at high stimulus levels LTG was affecting these kinetics. This interesting possibility has not been previously reported. Because this represents a potential new effect of LTG on the Na^+ channel, it would be of considerable value to establish if this effect could be consistently repeated by other workers. If this effect were found to be consistent then it would have further mechanistic implications for the actions of LTG on Na^+ channels that were previously unknown.

5.11.9. The potential significance of the action of LTG in understanding mechanisms underlying neuronal damage and performance deficit

The use of VDSCMs as tinnitolytics - One of the potential areas for further investigation arising from this current study is to look at VDSCMs in the treatment of tinnitus. LTG had previously been used as a putative tinnitolytic in a clinical study by Simpson *et al* (1999) motivated by the previous use of antiepileptics and intravenous lignocaine, both with sites of action at Na^+ channels. The use of these agents had met with some success though the specific site of action within the auditory pathway remained unknown. There was marginal evidence from the study by Simpson *et al* that LTG was successful in some patients, especially in those with long standing tinnitus. This probably reflected the mixed aetiology underlying the tinnitus of the patients as there did not appear to be any specific selection criteria in deciding what patients should be entered for the study.

The results gained in this chapter certainly support the idea that LTG acts at the level of the CN. Notwithstanding the mixed aetiology of tinnitus, the results may also help explain why there was only limited success with LTG. Tinnitus patients report a very wide range in the perceived loudness of their tinnitus from about 10-15 dB SPL up to 90-100 dB SPL though the majority of sufferers report levels up to 40 dB SPL (CIBA foundation symposium, 1981). If this tinnitus were generated at the level of the auditory nerve then, in those nerve fibres carrying this signal, this would represent only a low to moderate intensity suprathreshold signal thereby providing equivocal results with LTG.

The results shown in figures 5.10 to 5.13 suggest that LTG may have had a modest though non-significant, effect on the amplitude of the N_1 signal generation. Significant effects were, however, seen post LTG treatment on the EDAC power spectrum following noise insult (100 dB SPL) as shown in figures 5.28 and 5.29. This could be considered as a model for signal for the perception of a very loud broadband tinnitus-like signal. Whilst there was some attenuation of the neural signal by LTG this would not have represented a total suppression of the signal representing the tinnitus. This provides additional grounds for consideration of LTG and other VDSCMs as potential tinnitolytics.

As discussed previously, the action of VDSCMs is not dependent on discharge rate *per se,* but is principally determined by the level of depolarisation that accompanies neural activity (Xie *et al*, 1995). Therefore the potential for tinnitolytic (and antiepileptic) action of any VDSCM would expected to be related to the level of depolarisation that allowed it to bind to its active site. If this is correct then two inferences could be made about the potential use of VDSCMs as tinnitolytics. The first is that for LTG at least, it would be expected to be more effective in the more severe types of tinnitus. This kind of tinnitus would be expected to result in a greater degree of depolarisation in those neurones carrying the signal. The second inference is that other VDSCMs that bind at lower levels of discharge-dependent depolarisation would be more effective tinnitolytics. The tinnitolytic potential may also be enhanced by having a more prolonged inactivation time following binding.

The results presented in this chapter and the further development of ESAC/EDAC measurement within this thesis provide a basis for further experimental studies investigating the suitability of VDSCMs as tinnitolytics

Is the effect of LTG on high frequency (30 kHz) threshold and amplitude evidence of tonotopic differences in the resting potential? - The effect of LTG on thresholds and CAP amplitude at 30 kHz provided some intersupporting evidence of a possible tonotopic effect. This could be explained in terms of some biophysical difference in the CN simply affecting partitioning of LTG (Parsons *et al*, 1995). As suggested in section 5.4.3, it may reflect a greater effect of LTG in these fibres because they possess a higher interspike resting membrane potential. This in turn may reflect the recognised greater vulnerability of these fibres to a broader variety of insults. This tonotopic difference vulnerability is particularly well documented in the case of ototoxicity (Brummett and Fox, 1982, Stebbins *et al* 1981, Mulheran, 1990). Moreover the pattern of gradual loss of hearing follows this same tonotopic pattern from high to low frequency that characterises presbyacusis in man and other experimental mammals.

The possession of a hypothesised resting membrane potential nearer zero may be as a result of experiencing repeated moderate ototrauma. This would compromise the neurones ability to keep the resting potential at a more negative level. This in turn may reflect or lead to poorer Ca^{2+} homeostasis. If correct this idea could serve as an early electrophysiological marker for likely neuronal dysfunction or cell death throughout the rest of the nervous system.

This idea could also be expanded to explain some kinds of tinnitus that arise from a higher resting potential in neurones at any other tonotopic location in the cochlea. In this case, the higher resting potential could lead to an increase in spontaneous discharge in these fibres leading to the perception of tinnitus.

The CN as a separate site for noise damage; A comparison with findings of other workers - The involvement of the CN as a distinct site caused by noise as supported by the results presented here is a novel finding. This is in partial contrast to the findings reported by Puel *et al* (1988) discussed in some detail in Chapter 4.6. Puel *et al* used 6 kHz at 95dB SPL for 15 minutes in pigmented guinea pigs compared to the 100 dB SPL bandpass noise (about 5-20 kHz) in albino guinea pigs in this thesis. The authors reported similar peak threshold elevations as in this thesis, but found no evidence of post synaptic involvement as the effects of noise were just as marked with or without 5mM kynurenate perfusion. They proposed that most of the damage was physical in nature, occurring at

the level of the OHC. In a later study, however, they did report evidence of post-synaptic involvement at higher stimulus intensities from 100 to 130 dB SPL (Puel *et al,* 1998).

In this chapter, threshold elevation was shown to be significantly ameliorated by prior treatment with LTG, providing evidence not only of post-synaptic involvement but also that this the involvement was not excitotoxic in nature. The absence of this involvement at the lower intensity level in the studies by Puel's group could be explainable by differences in pigmentation or possibly difference in choice of insult, ie. single tone vs bandpass noise.

5.11.10. Future work

The results presented here provide a promising basis for the continued exploration of the physiology and pharmacology of cochleoprotection. Future studies emanating from this work are numerous and varied, but a number of options are provided below for consideration:

- Expose animals to NH in the presence of LTG. Because this insult was shown to be more severe than noise alone, this would demonstrate whether or not a use dependent inhibition was important in CN function.

- Increase the numbers of animals exposed to 12% hypoxia to ensure greater statistical power and further confidence in the data set.

- Utilise both light and electron microscopy techniques to ascertain for certain whether or not there are any physical changes following this acute noise/NH insult. Electron microscopy (transmission and surface) could help show any potential signs of excitotoxicity by the presence of vacuolation at the afferent nerve IHC synapse, as well as showing direct hair cell damage/loss.

- Monitor the recovery of the noise/NH changes over time. This would probably involve the use of surgically implanted electrodes to ensure accurate repetition of physiological recordings. This would help establish whether the CAP threshold shifts were temporary or permanent.

- Does anaesthesia really affect LTG distribution? A similar range of studies as performed in this thesis would need to be conducted in non-anaesthetised animals to confirm this.

- Identify the presence of glutamate release using analytical techniques such as enzymatic cycling procedures to help show whether or not LTG had modulated its release.

- Follow up experiments to see whether LTG is having an effect on normal cochlear function, in particular at high frequencies. This could be confirmed by ensuring

control recordings are taken prior to LTG being administered. A time course of recordings could then be performed to show any effects of LTG.

- In light of the potential threshold changes seen at 30 kHz following LTG administration, one of the intriguing possibilities to be flagged by this was that differences in neuronal resting potential might occur. This may act as a marker or be related to the degree of stress or trauma previously experienced. This possibility could be tested could directly in the CN by carrying out single unit recordings and measuring their resting potential. This could be then correlated against physiological parameters such as frequency, threshold, spontaneous and driven activity and refractory properties. This would provide some measure of functional status both in control and LTG treated fibres. The experimental groups could also comprise animals in different age groups to establish if resting potentials changed with age following acute or chronic noise insult ie. effects on the resting potential or acceleration of the ageing process.

- Use analogues of LTG to see whether there are better compounds in this class at offering protection against NIHL.

- Dose with LTG post noise/NH insult. This would have to be performed following a controlled recovery phase study but it would provide evidence of potential 'recovery therapy' for LTG and other VDSCMs.

- Administer LTG via the IntraEar Ecath® to see whether better protection against NIHL is provided. A dose response curve would need to be constructed around the LTG concentration identified in perilymph from this thesis. From these experiments, it would be possible to demonstrate that higher or lower concentrations of LTG could be delivered locally, thereby offering greater protection without the potential for systemic side effects.

- Co-administer LTG with agents that could potentially modulate lateral or medial efferent nerve fibre function, such as nicotinic, muscarinic or dopaminergic antagonists/agonists. This would demonstrate and potential synergy between such

agents. Both systemic and local administration studies utilising the IntraEar Ecath® would be valuable.

- Co-administer LTG with agents that could potentially modulate VGCCs to a great degree than LTG. Same reasons as above.

- Further investigate the change in power 'hump' observed in EDAC measurement following LTG/noise exposure. A range of VDSCMs/VDCCMs may help identify any potential changes in ion channel kinetics.

- Perform antibody studies to characterise the Na^+ channel populations present in the inner ear and CN. This will help identify potentially suitable VDSCMs as cochleoprotectants.

It is clear from a review of the literature that that there are a number of agents that could be considered as potential cochleoprotectants. However, the experience from this study provides grounds for considerable optimism leading to the early realisation that the cochlea is a target organ for therapeutic drugs in man.

CHAPTER 3. The effects of hypoxia, noise and noise/hypoxia combination on the BAER

BAER Threshold – within group ANOVA

Group	F_{df}	F value	P
Control	$F_{7,90}$	0.12	0.98
Noise (60 mins)	$F_{8,41}$	0.9	0.53
Noise (120 mins)	$F_{4,25}$	0.86	0.5
Hypoxia (60 mins)	$F_{8,69}$	1.32	0.25
Hypoxia (120 mins)	$F_{4,40}$	1.97	0.118
Noise/Hypoxia (60 mins)	$F_{8,42}$	1.59	0.17
Noise/Hypoxia (120 mins)	$F_{4,20}$	1.86	0.16
Amplitude at 40 dB HTL			
Group	*F_{df}*	*F value*	*P*
Control	$F_{7,88}$	0.13	0.99
Noise (60 mins)	$F_{8,45}$	0.93	0.5
Noise (120 mins)	$F_{7,40}$	0.49	0.836
Hypoxia (60 mins)	$F_{8,81}$	1.38	0.22
Hypoxia (120 mins)	$F_{4,45}$	1.7	0.17
NH (120 mins)	***$F_{8,42}$***	***4.76***	***<0.0001***
Amplitude at 80 dB HTL			
Group	*F_{df}*	*F value*	*P*
Control	$F_{7,88}$	0.32	0.94
Noise (60 mins)	***$F_{8,36}$***	***2.45***	***0.03***
Noise (120 mins)	***$F_{4,20}$***	***6.8***	***0.001***
Hypoxia (60 mins)	*$F_{8,78}$*	*0.41*	*0.91*
NH (120 mins)	***$F_{8,42}$***	***10.73***	***<0.0001***
ANOVA – Latency at 40 dB HTL			
Group	*F_{df}*	*F value*	*P*
Control	$F_{7,87}$	0.41	0.89
Noise (60mins)	$F_{8,35}$	0.57	0.53
Noise (120 mins)	$F_{7,31}$	0.41	0.89
Hypoxia (120 mins)	$F_{8,74}$	0.37	0.86
NH (120 mins)	$F_{8,35}$	***2.33***	***0.04***
ANOVA – Latency at 80 dB HTL			
Group	*F_{df}*	*F value*	*P*
Control	***$F_{7,79}$***	***5.28***	***<0.0001***
Noise (60 mins)	$F_{8,36}$	1.8	0.11
Noise (120 mins)	***$F_{4,20}$***	***3.9***	***0.016***
Hypoxia (120 mins)	***$F_{8,81}$***	***3.27***	***0.003***
NH (120 mins)	***$F_{8,43}$***	***13.61***	***<0.0001***

Chapter 4. The effects of noise, 12% hypoxia or noise/12% hypoxia on compound action potential (CAP) and ensemble spontaneous CN activity (ESAC)

CAP threshold – ANOVA
Artificial perilymph time control over 60 minutes

Frequency (kHz)	F_{df}	F value	P
8	$F_{5,36}$	0.07	0.996
16	$F_{5,36}$	0.09	0.993
24	$F_{5,38}$	0.06	0.998
30	$F_{5,38}$	0.0	1.00

Within group comparison **- control (T_0) pre noise and 15 min noise**

Frequency (kHz)	F_{df}	F value	P
8	$F_{3,19}$	68.91	<0.0001
16	$F_{3,24}$	59.18	<0.0001
24	$F_{3,24}$	12.05	<0.0001
30	$F_{3,24}$	0.57	0.643

t-test values comparing T_0 with 15mins noise all p<0.0001, except at 30kHz (P=0.298)

Within group comparison **- control (T_0) pre NH and 15 min NH**

Frequency (kHz)	F_{df}	F value	P
8	$F_{3,36}$	145.47	<0.0001
16	$F_{3,36}$	171.78	<0.0001
24	$F_{3,36}$	49.57	<0.0001
30	$F_{3,32}$	8.91	<0.0001

t-test values comparing T_0 with 15mins NH all p<0.0001

Across group comparison **– control, hypoxia, noise and NH**

Frequency (kHz)	F_{df}	F value	P
8	$F_{3,24}$	69.59	<0.0001
16	$F_{3,25}$	56.08	<0.0001
24	$F_{3,25}$	51.32	<0.0001
30	$F_{3,23}$	8.77	<0.0001

t-test values comparing T_0 with 15mins noise all p<0.0001, except at 30kHz (P=0.298)

Across group comparison **– pretreatment artificial perilymph control, hypoxia, noise or NH**

Frequency (kHz)	F_{df}	F value	P
8	$F_{3,22}$	1.75	0.187
16	$F_{3,25}$	1.87	0.161
24	$F_{3,25}$	0.39	0.765
30	$F_{3,21}$	0.11	0.955

CAP threshold t-test

	Inter group comparison (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	**<0.05**	**<0.0001**	**<0.0001**
16	non sig	**<0.0001**	**<0.0001**
24	non sig	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.01**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.0001**	**<0.0001**	**<0.01**
16	**<0.001**	**<0.0001**	**<0.05**
24	**<0.0001**	**<0.0001**	**<0.0001**
30	**<0.0001**	**<0.01**	non sig

CAP N_1 amplitude *within group comparison* – ANOVA
control (T_0) pre hypoxia and 15 min hypoxia (8 kHz)

dB attenuation	F_{df}	F value	P
0	$F_{2,8}$	0.03	0.972
20	$F_{2,8}$	0.88	0.452
40	$F_{2,6}$	2.02	0.213
60	$F_{2,6}$	0.91	0.451

control (T_0) pre noise and 15 min noise (8 kHz)

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,18}$	0.12	0.891	=0.891
20	**$F_{2,18}$**	**6.14**	**<0.01**	**<0.01**
40	**$F_{2,15}$**	**39.95**	**<0.0001**	**<0.0001**
60	no results due to threshold shifts			

control (T_0) pre NH and 15 min NH (8 kHz)

dB attenuation	F_{df}	F value	P	t-test P value
0	**$F_{2,23}$**	**11.69**	**<0.0001**	**<0.0001**
20	**$F_{2,22}$**	**25.64**	**<0.0001**	**<0.0001**
40	no results due to threshold shifts			
60				

***Across group comparison* – ANOVA control, hypoxia, noise and NH (8 kHz)**

dB attenuation	F_{df}	F value	P
0	**$F_{3,22}$**	**8.52**	**<0.001**
20	**$F_{3,22}$**	**22.65**	**<0.0001**
40	**$F_{3,21}$**	**49.37**	**<0.0001**
60	**$F_{3,21}$**	**14.26**	**<0.001**

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (16 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

control (T_0) pre noise and 15 min noise (16kHz)

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,18}$	4.71	<0.05	<0.01
20	$F_{2,18}$	18.56	<0.0001	<0.001
40	$F_{2,18}$	23.49	<0.0001	<0.0001
60	no results due to threshold shifts			

control (T_0) pre NH and 15 min NH (16 kHz)

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,23}$	14.95	<0.0001	<0.001
20	$F_{2,22}$	15.41	<0.0001	<0.001
40	$F_{2,23}$	18.58	<0.0001	<0.001
60	no results due to threshold shifts			

Across group comparison **control, hypoxia, noise and NH (16 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,22}$	35.72	<0.0001
20	$F_{3,22}$	27.02	<0.0001
40	$F_{3,22}$	62.21	<0.0001
60	$F_{3,22}$	51.88	<0.0001

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (24 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

Within group comparison **control (T_0) pre noise and 15 min noise (24 kHz)**

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,18}$	0.63	0.542	NS
20	$F_{2,18}$	3.16	0.067	<0.0001
40	$F_{2,18}$	4.14	<0.05	<0.0001
60	$F_{2,18}$	8.18	<0.01	<0.0001

Within group comparison
control (T_0) pre NH and 15 min NH (24 kHz)

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,22}$	6.80	<0.01	<0.0001
20	$F_{2,23}$	7.11	<0.01	<0.01
40	$F_{2,24}$	11.44	<0.0001	<0.001
60	$F_{2,22}$	11.80	<0.0001	<0.001

Across group comparison **control, hypoxia, noise and NH (24 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,22}$	17.06	<0.0001
20	$F_{3,22}$	13.63	<0.0001
40	$F_{3,22}$	20.10	<0.0001
60	$F_{3,22}$	9.05	<0.0001

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (30 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

Within group comparison **control (T_0) pre noise and 15 min noise (30 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{2,18}$	0.73	0.494
20	$F_{2,18}$	0.14	0.874
40	$F_{2,18}$	1.71	0.210
60	$F_{2,14}$	1.40	0.278

Within group comparison **control (T_0) pre NH and 15 min NH (30 kHz)**

dB attenuation	F_{df}	F value	P	t-test P value
0	$F_{2,22}$	4.26	<0.05	<0.05
20	$F_{2,21}$	5.09	<0.05	<0.05
40	$F_{2,20}$	5.42	<0.05	<0.01
60	$F_{2,18}$	8.98	<0.01	<0.01

Across group comparison **control, hypoxia, noise and NH (30 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,21}$	10.15	<0.0001
20	$F_{3,21}$	16.29	<0.0001
40	$F_{3,20}$	7.26	<0.01
60	$F_{3,20}$	6.50	<0.01

CAP N_1 amplitude
Across group comparison t-test

	0 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	non sig	non sig	**<0.002**
16	non sig	**<0.01**	**<0.0001**
24	non sig	non sig	**<0.0001**
30	non sig	non sig	**<0.01**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	non sig	**<0.01**	**<0.0001**
16	**<0.0001**	**<0.0001**	**<0.001**
24	non sig	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.01**
	20 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	non sig	**<0.0001**	**<0.0001**
16	non sig	**<0.007**	**<0.0001**
24	non sig	non sig	**<0.001**
30	0.112	**<0.05**	**<0.01**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.05**	**<0.001**	**<0.01**
16	**<0.0001**	**<0.0001**	**<0.0001**
24	non sig	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.0001**
	40 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	non sig	**<0.0001**	**No data**
16	non sig	**<0.0001**	**<0.0001**
24	non sig	non sig	**<0.0001**
30	non sig	non sig	**<0.05**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.0001**	No results due to threshold shifts	
16	**<0.0001**	**<0.0001**	**<0.01**
24	non sig	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.01**
	60 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8 and 16	No results due to threshold shifts		
24	non sig	non sig	**<0.01**
30	non sig	non sig	**<0.05**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8 and 16	No results due to threshold shifts		
24	**<0.05**	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.05**

CAP N_1 latency – ANOVA
Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (8 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,11}$	0.39	0.759
20	$F_{3,11}$	0.06	0.980
40	$F_{3,7}$	0.72	0.570
60	$F_{3,8}$	0.29	0.833

Within group comparison **control (T_0) pre noise and 15 min noise (8 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,24}$	16.59	<0.0001
20	$F_{3,24}$	20.82	<0.0001
40	$F_{3,18}$	84.87	<0.0001
60	no results due to threshold shifts		

Within group comparison **control (T_0) pre NH and 15 min NH (8 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,31}$	106.42	<0.0001
20	$F_{3,30}$	102.15	<0.0001
40 and 60	no results due to threshold shifts		

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (16 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

Within group comparison **control (T_0) pre noise and 15 min noise (16 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,24}$	2.30	0.103
20	$F_{3,24}$	0.85	0.482
40	$F_{3,23}$	13.00	<0.0001
60	no results due to threshold shifts		

Within group comparison **control (T_0) pre NH and 15 min NH (16 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,31}$	1.24	0.311
20	$F_{3,30}$	14.28	<0.0001
40	$F_{3,27}$	36.30	<0.0001
60	no results due to threshold shifts		

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (24 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

Within group comparison **control (T_0) pre noise and 15 min noise (24 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,24}$	0.86	0.477
20	$F_{3,24}$	0.81	0.50
40	$F_{3,24}$	1.22	0.324
60	**$F_{3,24}$**	**6.68**	**<0.01**

Within group comparison **control (T_0) pre NH and 15 min NH (24 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,31}$	1.37	0.270
20	**$F_{3,31}$**	**4.36**	**<0.05**
40	**$F_{3,30}$**	**8.01**	**<0.0001**
60	$F_{3,25}$	1.83	0.167

Within group comparison **control (T_0) pre hypoxia and 15 min hypoxia (30 kHz)**

dB attenuation	F_{df}	F value	P
0	No significant effects at any dB attenuation		
20			
40			
60			

Within group comparison **control (T_0) pre noise and 15 min noise (30 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,24}$	0.43	0.730
20	$F_{3,24}$	0.45	0.718
40	$F_{3,24}$	0.04	0.990
60	$F_{3,20}$	0.66	0.585

Within group comparison **control (T_0) pre NH and 15 min NH (30 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,31}$	0.24	0.869
20	$F_{2,20}$	0.00	0.997
40	$F_{3,27}$	0.75	0.533
60	$F_{3,21}$	1.53	0.235

Across group comparison **control, hypoxia, noise and NH (8 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,22}$	39.57	<0.0001
20	$F_{3,22}$	53.25	<0.0001
40	$F_{2,9}$	39.58	<0.0001
60	Insufficient results for across group analysis		

Across group comparison **control, hypoxia, noise and NH (16 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,23}$	3.18	<0.05
20	$F_{3,22}$	5.73	<0.01
40	$F_{3,17}$	17.97	<0.0001
60	no results due to threshold shifts		

Across group comparison **control, hypoxia, noise and NH (24 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,22}$	1.90	0.159
20	$F_{3,21}$	4.09	<0.05
40	$F_{3,19}$	5.63	<0.01
60	$F_{2,11}$	4.92	<0.05

Across group comparison **control, hypoxia, noise and NH (30 kHz)**

dB attenuation	F_{df}	F value	P
0	$F_{3,21}$	0.34	0.796
20	$F_{3,19}$	0.52	0.672
40	$F_{3,16}$	1.47	0.261
60	$F_{3,12}$	2.09	0.156

CAP N_1 Latency t-test *across group comparison*

	0 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	**<0.05**	**<0.0001**	**<0.0001**
16	non sig	**<0.0001**	**<0.0001**
24	non sig	**<0.0001**	**<0.0001**
30	non sig	**<0.0001**	**<0.01**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.0001**	**<0.0001**	**<0.01**
16	**<0.001**	**<0.0001**	**<0.05**
24	**<0.0001**	**<0.0001**	**<0.0001**
30	**<0.0001**	**<0.01**	non sig
	20 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	**0.327**	**<0.0001**	**<0.0001**
16	non sig	non sig	**<0.01**
24	non sig	non sig	non sig
30	non sig	non sig	non sig
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.01**	**<0.0001**	**<0.01**
16	non sig	**<0.05**	non sig
24	non sig	**<0.05**	**<0.05**
30	non sig	non sig	non sig
	40 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	non sig	**<0.0001**	**threshold shift**
16	non sig	**<0.0001**	**<0.0001**
24	non sig	non sig	non sig
30	non sig	non sig	**<0.05**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8	**<0.01**	no results due to threshold shifts	
16	**<0.05**	**<0.05**	**<0.05**
24	non sig	**<0.01**	non sig
30	non sig	**<0.05**	non sig
	60 dB attenuation (p value)		
Frequency (kHz)	**control vs hypoxia**	**control vs noise**	**control vs NH**
8	non sig	no results due to threshold shifts	
16	non sig	no results due to threshold shifts	
24	non sig	non sig	no res. threshold shifts
30	non sig	non sig	**<0.05**
	hypoxia vs noise	**hypoxia vs NH**	**noise vs NH**
8 and 16	no results due to threshold shifts		
24	**<0.05**	no results due to threshold shifts	
30	non sig	**<0.05**	non sig

ESAC power (0.5 to 1.5 kHz) – ANOVA
Within group comparison **Artificial perilymph time control over 60 minutes**

F_{df}	F value	P
$F_{12,62}$	0.79	=0.661

Within group comparison **control (T_0) pre and post 15 minutes hypoxia**

F_{df}	F value	P
$F_{10,20}$	1.52	=0.203

Within group comparison **control (T_0) pre and post 15 minutes noise**

F_{df}	F value	P
$F_{10,50}$	**376.49**	**<0.0001**

Within group comparison **control (T_0) pre and post 15 minutes NH**

F_{df}	F value	P
$F_{11,58}$	**198.27**	**<0.0001**

ESAC/EDAC power change (0.5 to 1.5 kHz)
Student's t-test control vS either 15 minutes noise, 12% hypoxia or NH

Time of noise exposure (minutes)	Hypoxia	Noise	NH
0	non sig	**<0.0001**	**<0.0001**
5	**<0.05**	**<0.0001**	**<0.0001**
10	non sig	**<0.0001**	**<0.0001**
15	**<0.05**	**<0.0001**	**<0.0001**
16 (post exposure)	non sig	**<0.01**	**<0.0001**
25	non sig	**<0.01**	**<0.001**
30	no data	no data	**<0.01**
35	no data	no data	**<0.01**

Chapter 5. The effects of systemic lamotrigine (LTG) on protection against noise induced hearing loss (NIHL)

Across group comparison **pharmacokinetics**

	F_{df}	F value	P
Perilymph	$F_{2,9}$	**46.27**	**<0.0001**
Plasma	$F_{2,12}$	2.4	=0.133

Perilymph pharmacokinetics t-test

	unsedated LTG/sedated LTG	unsedated LTG/noise control	LTG control/noise control
Perilymph	**p<0.001**	**p<0.0001**	=0.131

Across group comparison **Threshold – Pretreatment control, noise control, LTG control, noise/LTG control**

Frequency (kHz)	F_{df}	F value	P
8	$F_{3,28}$	0.55	=0.652
16	$F_{3,30}$	0.28	=0.842
24	$F_{3,30}$	0.97	=0.422
30	$F_{3,30}$	1.82	=0.164

Threshold t-test – noise control compared to noise/LTG

Frequency (kHz)	p value
8	**<0.05**
16	=0.11
24	=0.624
30	**<0.05**

CAP N_1 amplitude ANOVA
control, 15 min noise, 15 min noise/LTG

0dB attenuation			
Frequency (kHz)	**F_{df}**	**F value**	**P**
8	$F_{2,15}$	**7.04**	**<0.01**
16	$F_{2,16}$	**4.38**	**<0.05**
24	no significance		
30	no significance		
20dB attenuation			
	F_{df}	**F value**	**P**
8	$F_{2,15}$	**9.3**	**<0.01**
16	$F_{2,16}$	**7.77**	**<0.01**
24	no significance		
30	no significance		
40dB attenuation			
	F_{df}	**F value**	**P**
8	$F_{2,14}$	**32.72**	**<0.0001**
16	$F_{2,15}$	**14.69**	**<0.0001**
24	no significance		
30	no significance		
60dB attenuation			
	F_{df}	**F value**	**P**
8	$F_{1,8}$	**9.57**	**<0.05$^{\$}$**
16	$F_{1,8}$	**9.57**	**<0.05$^{\$}$**
24	no significance		
30	no significance		

$ *Data available between control and 15 minutes LTG/noise only*

CAP N_1 Amplitude t-test
control compared to 15 min noise or 15 min noise/LTG

Frequency	noise	noise/LTG
8 kHz		
0	<0.05	non sig
20	<0.01	<0.01
40	<0.0001	<0.0001
60	no data	no data
16 kHz	noise	noise/LTG
0	<0.05	<0.0001
20	<0.01	<0.05
40	<0.05	<0.01
60	no data	<0.05
24 kHz	noise	noise/LTG
0	non sig	non sig
20	non sig	non sig
40	non sig	non sig
60	<0.05	<0.01
30 kHz	noise	noise/LTG
0	non sig	<0.05
20	non sig	non sig
40	non sig	non sig
60	non sig	non sig

CAP N_1 latency ANOVA
control, 15 min noise, 15 min noise/LTG

0dB attenuation			
Frequency	**F_{df}**	**F value**	**P**
8	$F_{2,18}$	**16.06**	**<0.0001**
16	non sig		
24	non sig		
30	$F_{2,18}$	**4.46**	**<0.05**
20dB attenuation			
Frequency	**F_{df}**	**F value**	**P**
8	$F_{2,17}$	**17.16**	**<0.0001**
16	non sig		
24	$F_{2,18}$	**5.45**	**<0.05**
30	$F_{2,18}$	**3.95**	**<0.05**
40dB attenuation			
Frequency	**F_{df}**	**F value**	**P**
8	$F_{2,14}$	**57.04**	**<0.0001**
16	$F_{2,17}$	**7.13**	**<0.01**
24	$F_{2,18}$	**8.87**	**<0.01**
30	non sig		
60dB attenuation			
Frequency	**F_{df}**	**F value**	**P**
8	**No data**		
16*	$F_{1,9}$	**12.12**	**<0.01**
24	$F_{2,16}$	**10.02**	**<0.001**
30	non sig		

* No data for 15 minute noise exposure

CAP N_1 latency t-test
control compared to 15 min noise or 15 min noise/LTG

8 kHz	noise	noise/LTG
0	<0.0001	<0.0001
20	<0.0001	<0.0001
40	<0.0001	<0.0001
60	no data	no data
16 kHz	noise	noise/LTG
0	non sig	non sig
20	non sig	non sig
40	<0.001	<0.01
60	no data	<0.01
24 kHz	noise	noise/LTG
0	non sig	non sig
20	non sig	<0.05
40	non sig	<0.01
60	<0.01	<0.0001
30 kHz	noise	noise/LTG
0	non sig	<0.05
20	non sig	<0.05
40	non sig	non sig
60	non sig	non sig

APPENDIX 2

PATENT APPLICATION

The use of frequency dependent voltage activated sodium channel blockers particularly lamotrigine in the prevention of noise induced hearing loss

International publication number WO 02/28394 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number
WO 02/28394 A1

(51) International Patent Classification[7]: A61K 31/53

(21) International Application Number: PCT/EP01/11498

(22) International Filing Date: 5 October 2001 (05.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0024517.5 6 October 2000 (06.10.2000) GB

(71) Applicant *(for all designated States except US)*: **GLAXO GROUP LIMITED** [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **ETHERIDGE, Steven** [GB/GB]; c/o GlaxoSmithKline, Park Road, Ware, Hertfordshire, SG12 0DP (GB).

(74) Agent: **HOCKLEY, Sian, Catherine**; Corporate Intellectual Property, GlaxoSmithKline, 980 Great West Road, Brentford, Middlesex TW8 9GS (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF MEDICAMENTS

(57) Abstract: The present invention provides the use of frequency dependent voltage activated sodium channel blockers particularly lamotrigine in the prevention of noise induced hearing loss.

WO 02/28394 A1

Use of Medicaments

The present invention relates to a new use of frequency dependent voltage activated sodium channel blockers. In particular, the present invention relates to a new use of 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine and its pharmaceutically acceptable acid addition salts.

Compounds exhibiting frequency dependent voltage activated sodium channel blocker activity include those described in EP-A-0021121, WO97/09317, WO98/38174, WO99/32462 and WO00/12488.

Simpson *et al.*, The Assessment of Lamotrigine, an Antieplileptic Drug, in The Treatment of Tinnitus, *Am J Otol* 1999; 20: p 627-631 suggests that lamotrigine might be useful in the treatment of tinnitus.

NIHL is the most frequent occupational disease with prevalence of 30 to 50% in exposed populations. At present the only effective method for preventing NIHL is for the subject to wear some form of physical shield, such as ear protectors. However, ear protectors are only of use when the subject is aware the he is likely to be exposed to a noise insult and in some circumstances, such as in a battlefield situation, exposure to a noise insult can be sudden and unexpected. Moreover, the use of ear protectors affects the subjects hearing across its whole range often making quiet noises, such as conversation, inaudible. Accordingly, there is a need for alternative means for protecting the ear against noise induced hearing loss which does not affect normal auditory function. In particular, there is a need for protecting the ear against hearing loss resulting from acute or chronic noise induced hearing loss which does not affect normal auditory function.

It has now been surprisingly found that frequency dependent voltage activated sodium channel blockers, particularly 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine and its pharmaceutically acceptable acid addition salts, are effective in the prevention of noise induced hearing loss (NIHL).

Accordingly, the present invention provides the use of a frequency dependent voltage activated sodium channel blocker or a pharmaceutically acceptable acid addition salt thereof in the manufacture of a medicament for the prevention of noise induced hearing loss (NIHL).

Brief description of the figures

Figure 1 illustrates the pharmacokinetic profile of 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine in plasma and perilymph.

Figure 2 demonstrates the effect of 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine on cochlea nerve compound action potential threshold shifts in the absence of auditory stimulation.

Figure 3 illustrates the effect 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine on cochlea nerve compound action potential threshold shifts in response to discrete acoustic stimuli (110dB SPL).

Figure 4 demonstrates the effect of 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine on the gross ensemble spontaneous activity of the cochlea nerve fibre before during and after noise exposure (110dB spl).

3,5-Diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine will herein after be referred to as lamotrigine.

The term prevention as used herein with reference to prevention of noise induced hearing loss (NIHL) means to preclude, or ameliorate the progression of hearing loss.

The term noise induced hearing loss (NIHL) as used herein is intended to refer to the loss of hearing performance, marked by a loss of sensitivity and resolution in hearing resulting from over-stimulation of the auditory system. In particular, the term NIHL is intended to refer to the loss in hearing performance resulting from damage to the cochlear nerve as a result of prolonged or acute exposure to noise stimuli.

It is thought that voltage activated sodium channel blockers act by interacting with voltage activated sodium channels in such a way as to reduce the flow of sodium current when these channels are open. The term frequency dependent as used herein with reference to voltage activated sodium channel blockers is intended to cover those compounds whose interaction with the sodium channels is dependent on the rate the channels open and subsequently inactivate. Thus the effect of frequency dependent voltage activated sodium channel blockers will vary depending on the frequency with which the sodium channels open and inactivate.

Determination of the frequency dependence of a voltage activated sodium channel blocker requires sodium channels to be repetitively activated at a range of frequencies, in the presence and absence of the blocker. This may be achieved by applying depolarising pulses to elicit sodium currents using the whole-cell voltage-clamp technique and recording from a cell type containing voltage-activated sodium channels, for example Chinese hamster ovary cells expressing recombinant human brain type IIA sodium channels. As the frequency of the depolarisations is increased the degree of blockade increases, i.e. frequency dependence. Conversely, non-frequency dependent blockers will produce the same level of blockade for the complete range of frequencies applied. For a more detailed description of determination of frequency dependence see Ragsdale DS, Scheuer T, Catterall WA. Frequency and voltage-dependent inhibition of type IIA Na+ channels, expressed in a mammalian cell line, by local anesthetic, antiarrhythmic, and anticonvulsant drugs. *Mol Pharmacol* 1991 Nov;40(5):756-65.

Suitable examples of frequency dependent voltage activated sodium channel blockers include those described in EP-A-0021121, WO97/09317, WO98/38174, WO99/32462 and WO00/12488 all incorporated herein by reference. A suitable compound described in EP-A-0021121 is lamotrigine and pharmaceutically acceptable acid addition salts thereof. A suitable compound described in WO97/09317 is R(-)-2,4-diamino-5-(2,3-dichlorophenyl)-6-fluoromethyl pyrimidine and pharmaceutically acceptable acid addition salts thereof. A suitable compound described in WO98/38174 is 3-(2,3,5-trichloro-phenyl)-

pyrazine-2,6-diamine and pharmaceutically acceptable acid addition salts thereof. A suitable compound described in WO99/32462 is 5-amino-6-[2,3,5-trichlorophenyl]-1,2,4-triazine and pharmaceutically acceptable acid addition salts thereof. A suitable compound described in WO00/12488 is 2,6-diamino-5-carboxamido-3-(2,3,5-trichlorophenyl)pyrazine and pharmaceutically acceptable acid addition salts thereof. A preferred frequency dependent voltage activated sodium channel blocker is lamotrigine and its pharmaceutically acceptable acid addition salts.

Suitable pharmaceutically acceptable acid addition salts include those formed with both organic and inorganic acids. Examples of such salts include those formed with hydrochloric, sulphuric, citric, tartaric, phosphoric, lactic, pyruvic, acetic, succinic, fumaric, maleic, methanesulphonic, ethanesulphonic, oxaloacetic and isethionic acids.

As reported in the Examples that follow the otoprotective effect of the frequency dependent voltage activated sodium channel blocker, lamotrigine, against acute noise exposure was determined in the guinea pig model.

The experimental results presented herein demonstrate penetration of the guinea pig cochlea by lamotrigine. The absence of an effect of lamotrigine on the normal sensitivity of the cochlea over 8-30 kHz together with the absence of an effect on the normal gross spontaneous activity of the cochlear nerve, as measured by analysis of cochlear spontaneous noise signal, demonstrates that in the absence of traumatic noise lamotrigine has no discernible effect on normal auditory function.

The results further demonstrate that the elevation in threshold (the level of sound that was required to generate the smallest detectable electrophysiological response) caused by acute exposure to noise can be ameliorated if lamotrigine is administered prior to noise exposure. Moreover, the results establish that administration of lamotrigine prior to noise exposure can reduce the total amount of driven activity in the cochlear nerve. Suitably, the total amount of driven

activity in the cochlear nerve is reduced by more than 30%, preferably by more than 50%.

Without wishing to be bound by theory, it is believed that lamotrigine acts at the voltage activated sodium channels in the cochlear nerve by reducing the number of channels in the open state in a frequency dependent manner. It is thought to do achieve this by stabilising and prolonging the inactivation state of these channels. This reduces over stimulation of cochlear nerve fibres and thus prevents or ameliorates excitotoxic damage to these nerve fibres.

Accordingly, the present invention further provides a method of preventing noise induced hearing loss in a patient suffering from, or susceptible to, said disorder, which method comprises administering to the patient a therapeutically effective amount of a frequency dependent voltage activated sodium channel blocker or pharmaceutically acceptable acid addition salts thereof.

Compounds for use in the invention may be administered at a dose of from 0.1 to 40 mg/kg body weight per day, suitably 0.3 to 30 mg/kg body weight per day and more particularly 1 to 10 mg/kg weight per day, calculated as the free base. The dose range for adult human beings is generally from 8 to 2000 mg/day, such as from 35 to 1200 mg/day, preferably 10 to 500 mg/day or 20 to 200 mg/day, calculated as the free base. A particularly suitable dose range for adult human beings for lamotrigine is 100-400 mg/day.

While it is possible for the compounds to be administered as the raw chemical, it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise a frequency dependent voltage activated sodium channel blocker, such as lamotrigine and its pharmaceutically acceptable acid addition salts thereof, together with one or more acceptable carriers or diluents therefor and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The formulations include those suitable for oral, parenteral (including subcutaneous e.g. by injection or by depot tablet, intradermal, intrathecal, intramuscular e.g. by depot and intravenous), rectal and topical (including dermal, buccal and sublingual) administration and intracochlear perfusion / infusion although the most suitable route may depend upon for example the condition of the recipient. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the compounds ("active ingredient") with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include

suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of a sterile liquid carrier, for example, water-for-injection, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration may be presented as a suppository with the usual carriers such as cocoa butter, hard fat or polyethylene glycol.

Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges comprising the active ingredient in a flavoured basis such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a basis such as gelatin and glycerin or sucrose and acacia.

The compounds of the invention may also be formulated as depot preparations. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds of the invention may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In addition to the ingredients particularly mentioned above, the formulations may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

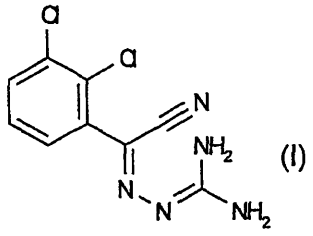
The compounds may be used in combination with other therapeutic agents, for example other frequency dependent voltage activated sodium channel blockers. When compounds are used in combination with other therapeutic agents, the compounds may be administered either sequentially or simultaneously by any convenient route. The invention thus provides, in a further aspect, a combination comprising a frequency dependent voltage activated sodium

channel blocker or a pharmaceutically acceptable derivative thereof with a further therapeutic agent for the prevention of noise induced hearing loss.

The combinations referred to above may conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical formulations comprising a combination as defined above together with a pharmaceutically acceptable carrier or excipient comprise a further aspect of the invention. The individual components of such combinations may be administered either sequentially or simultaneously in separate or combined pharmaceutical formulations.

R(-)-2,4-diamino-5-(2,3-dichlorophenyl)-6-fluoromethyl pyrimidine may be prepared according to the Examples described in WO97/09317. 3-(2,3,5-trichloro-phenyl)-pyrazine-2,6-diamine may be prepared according to the methods described in WO98/38174, see particularly Example 1. 5-amino-6-[2,3,5-trichlorophenyl]-1,2,4-triazine may be prepared according to the methods described in WO99/32462, see particularly the Example. 2,6-diamino-5-carboxamido-3-(2,3,5-trichlorophenyl)pyrazine may be prepared according to the methods described in WO00/12488, see particularly Example 1.

Lamotrigine may be prepared by a process which comprises cyclising the compound of formula (I):



(I)

and, if desired, converting lamotrigine thus obtained into a pharmaceutically acceptable acid addition salt.

The cyclisation is typically carried out by heating the compound of formula (I) under reflux in an alkanol, preferably a C_{1-4} alkanol, for example methanol or ethanol, in the presence of a strong base, for example potassium hydroxide.

The process may, for instance, be carried out as described in Example 1 of EP-A-0021121. The optional subsequent step of converting the lamotrigine into an acid addition salt is performed by a conventional method, for example by treatment with the appropriate acid at ambient temperature. The salt with isethionic acid may be prepared, for instance, as described in EP-A-0 247 892, in particular in Example 3. The starting compound of formula (I) may be prepared by the method described in US patent 3 637 688.

The invention is further illustrated in the Examples which follow.

Biological Examples

Experimental aim

The aim of these studies was to establish whether lamotrigine (LTG) would have any otoprotective effect against acute noise exposure in a guinea pig model.

Justification of LTG dose level

Data in the rat has shown an effective oral dose (ED_{50}) of 6.8mg/kg in the supramaximal electroshock (MES) model. Based on this data and pharmacokinetic data in the guinea pig (Parsons et al, 1995) a single oral dose of 20mg/kg (4mg/mL) in sterile water for irrigation was administered. All solutions were prepared fresh on the day of dosing and stored at 2-8°C, protected from light, between dosing sessions.

Surgical procedure

Sedation was achieved using an intramuscular injection of 0.5mL/kg medetomidine followed by an intraperitoneal injection of 6mL/kg fentanyl approximately 10 minutes later (loss of the pedal reflex was used to determine sufficient sedation for surgery). Supplementary doses of both agents were administered as necessary during the experiment.

The animal was placed in a prone position (left ear upwards) in a purpose built perspex chamber for the duration of the experiment. Core body temperature was maintained at 37°C +/- 1°C and a non-invasive pulse oximeter was placed on the

forepaw to measure blood oxygenation and heart rate. Both were kept within normal limits for the guinea pig throughout each experiment.

A combination recording electrode and drug delivery catheter was placed through the bulla into the round window niche with the aid of a surgical microscope. A cotton wick was placed around the drill hole to prevent body fluids from entering the middle ear cavity. Cochlear signals were then amplified (x 100 000) and bandpass filtered (0.1KHz-5KHz) for subsequent electrophysiological recording. No animal with any evidence of infection or abnormal development was used in the experiment.

Auditory assessment

The following two auditory recording techniques were used to investigate the potential otoprotective effects of Lamotrigine (LTG):

i) Recording system for gross ensemble spontaneous activity of cochlea nerve activity (ESAC)

Measurement of the gross ensemble spontaneous activity (ESAC) of cochlea nerve (CN) fibres was carried out by performing a Fast Fourier Transform (FFT) on the time signal generated by the cochlea. This was fed from the amplifier to a PC. The power spectra were then averaged to obtain the average spectrum of CN activity, expressed as $\mu V\ rms^2/Hz$. The difference in power between ESAC spectra was calculated in dB [dB=10 log (P1/P2)] where P1 is the pre-treatment FFT and P2 is a post treatment FFT over a given frequency range.

ii) Recording system for compound action potentials (CAP)

The cochlea nerve compound action potential (CAP) was generated in response to discrete acoustic stimuli. It was used to determine both control and treatment related changes in auditory thresholds by measuring changes in CAP latency (milliseconds) and amplitude (μVolts).

Tone 'pip' stimuli of 5 millisecond duration were computer generated at 8, 16, 24 and 30KHz and fed to an attenuator (1dB resolution). The tone pip was then fed to a Bruel and Kjaer 4192, (microphone) serving as an acoustic driver, which was placed inside an ear speculum coupled to the ear canal. CAP averaging

was performed between x50 to 100 times and the CAP threshold was determined by visual inspection of the recorded time signal. Accuracy was typically between 1 - 3dB, using a CAP detection criterion of approximately 1μV.

Example 1. The assay of plasma and perilymph for Lamotrigine (LTG)

In order to establish the otoprotective properties of LTG, a pharmacokinetic evaluation study was designed to establish penetration of LTG into guinea pig perilymph and plasma. A total of 17 healthy adult male albino guinea pigs were used. Plasma and perilymph samples were taken from 3 animals at each timepoint: 1, 2, 5, 10 and 15 hours after dosing. Two untreated control animals were also sampled for comparison.

Example 1. Experimental design

Heparinised blood samples (up to 10mL) for plasma were taken either via cardiac puncture or exsanguination via the jugular vein and frozen (-20°C) for subsequent analysis. After the terminal blood sample had been taken, the animal received an overdose of anaesthetic and was decapitated. The bulla was removed and thoroughly cleaned to remove all tissue and fluids. A glass micropipette was then used to puncture the round window membrane and, using gentle aspiration, approximately 6μL of perilymph was sampled and frozen (-20°C) for subsequent analysis. Levels of LTG in both plasma and perilymph were determined using a protein precipitation extraction procedure using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The LTG retention time was 5.4 minutes and quantification was carried out using peak areas.

Example 1. Results

Lamotrigine (LTG) was detected in both plasma and perilymph indicating good systemic exposure and penetration into the inner ear (Figure 1). Based on the timepoints used in this study, the levels of LTG detected in perilymph were approximately twice those seen in plasma with an observed mean maximum concentration (Cmax) of 6.14μg/mL and an approximate mean time of maximum concentration (Tmax) of 2 hours (mean plasma Cmax value was 3.59μg/mL with a Tmax of 1 hour). These results show the preferential uptake of LTG by the target organ (the cochlea). Mean plasma and perilymph LTG levels remained

relatively high after the 15 hour sampling period (1 and 3.2 μg/mL respectively) indicating comparable elimination rates.

Example 2. The effect of Lamotrigine (LTG) on normal auditory function

Example 2. Experimental design

To determine the effects of LTG on normal auditory function, a total of 20 healthy adult male albino guinea pigs were divided into two groups: Control (n=9) and 20mg/kg LTG (n=11) administered approximately 2 hours before surgery.

CAP thresholds at 8, 16, 24 and 30KHz were measured immediately after surgery and again approximately 15 minutes later.

Example 2. Results

Figure 2 shows the effect of LTG on CAP threshold shifts at the four frequencies measured (8, 16, 24 and 30KHz). There were no statistically significant differences between control and LTG treated animals at any of the 4 frequencies tested.

Example 3. Lamotrigine (LTG) otoprotection after acute noise insult

Example 3. Experimental design

A total of 14 healthy adult male albino guinea pigs were divided into two equal groups: Control (noise alone) and 20mg/kg LTG (administered approximately 2 hours before noise exposure).

CAP thresholds and ESAC were measured immediately after surgery and again approximately 15 minutes later. Animals were then exposed to 15 minutes filtered (5-20KHz) free field noise (approximately 110dB SPL) during which ESAC measurements were made at 5 minute intervals. Post noise CAP threshold and ESAC measurements were taken and the animal was then sacrificed.

Example 3. Results

Figure 3 shows the effect of LTG on CAP threshold shifts at the four frequencies measured (8, 16, 24 and 30KHz). The appearance of the threshold shift plot reflects the difference of spectral intensity of the filtered noise stimulus (maximum noise stimulus intensity was generated towards 8KHz to 16KHz). In all cases mean thresholds were higher in the LTG treated animals and reached statistical significance at 8 kHz ($p<0.05$).

Figure 4 shows the effect of LTG on ESAC before during and after noise exposure (only 6 LTG treated animals were used for ESAC measurement). Compared to noise control, a statistically significant decrease ($P<0.0001$) in the power spectrum of approximately 2dB was noted in the 20mg/kg LTG group during noise exposure. This significant decrease continued during the post noise exposure period ($p<0.0001$). No differences were noted between the two groups prior to noise exposure.

References

Catlin, F. I. (1986). Noise-induced hearing loss [Review]. American Journal of Otology **7**, 141-149.

Davis, A.C. (1989). The prevalence of hearing impairment and reported hearing disability among adults in Great Britain. International Journal of Epidemiology (18) 4, 911-917.

Parsons, D.N., Dickins, M and Morley, T. (1995). Lamotrigine: Absorption, distribution and excretion. Antiepileptic drugs, Fourth Edition, Raven Press Ltd., New York.

Example Pharmaceutical Compositions

Example 4: Lamotrigine Pharmaceutical Composition

Lamotrigine tablets for oral administration may formulated with the following ingredients:

Lamotrigine	150 mg
Lactose	200 mg
Maize starch	50 mg

Polyvinylpyrrolidone	4 mg
Magnesium stearate	4 mg

Mix the active compound with the lactose and starch and granulate with a solution of the polyvinylpyrrolidone in water. Dry the resulting granules, mix with the magnesium stearate and compress to give tablets of average weight 408 mg.

The application of which this description and claims forms part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process or use claims and may include, by way of example and without limitation, one or more of the following claims:

Claims

1. Use of a frequency dependent voltage activated sodium channel blocker or a pharmaceutically acceptable acid addition salt thereof in the manufacture of a medicament for the prevention of noise induced hearing loss.

2. Use according to claim 1 wherein the frequency dependent voltage activated sodium channel blocker is lamotrigine or a pharmaceutically acceptable acid addition salt thereof.

3. A method of preventing noise induced hearing loss in a patient suffering from, or susceptible to, said disorder, which method comprises administering to the patient a therapeutically effective amount of a frequency dependent voltage activated sodium channel blocker or pharmaceutically acceptable acid addition salt thereof.

4. A method according to claim 3 wherein the frequency dependent voltage activated sodium channel blocker is lamotrigine or a pharmaceutically acceptable acid addition salt thereof.

5. A pharmaceutical formulation comprising a frequency dependent voltage activated sodium channel blocker or a pharmaceutically acceptable acid addition salt thereof, together with one or more acceptable carriers or diluents therefor for use in the prevention of noise induced hearing loss.

6. A pharmaceutical formulation according to claim 5 wherein the frequency dependent voltage activated sodium channel blocker is lamotrigine or a pharmaceutically acceptable acid addition salt thereof.

FIG. 1

Pharmacokinetic profile of LTG in plasma and perilymph

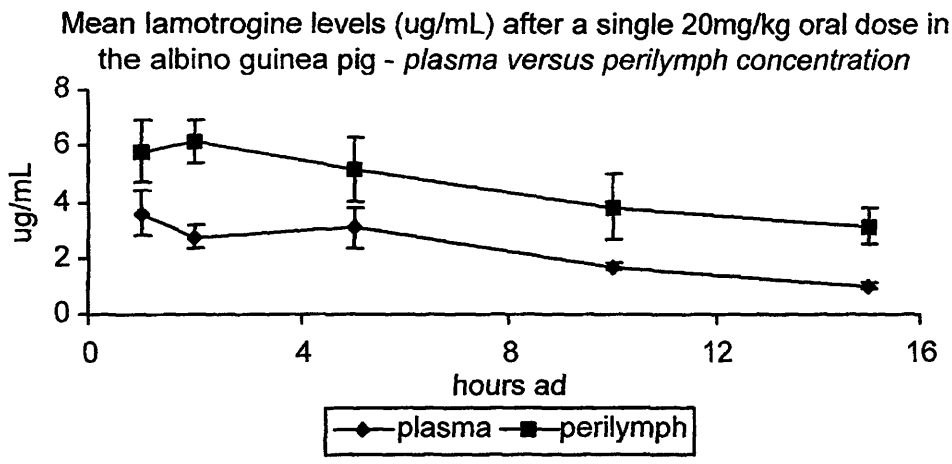


FIG. 2

The effect of LTG on CAP threshold shifts

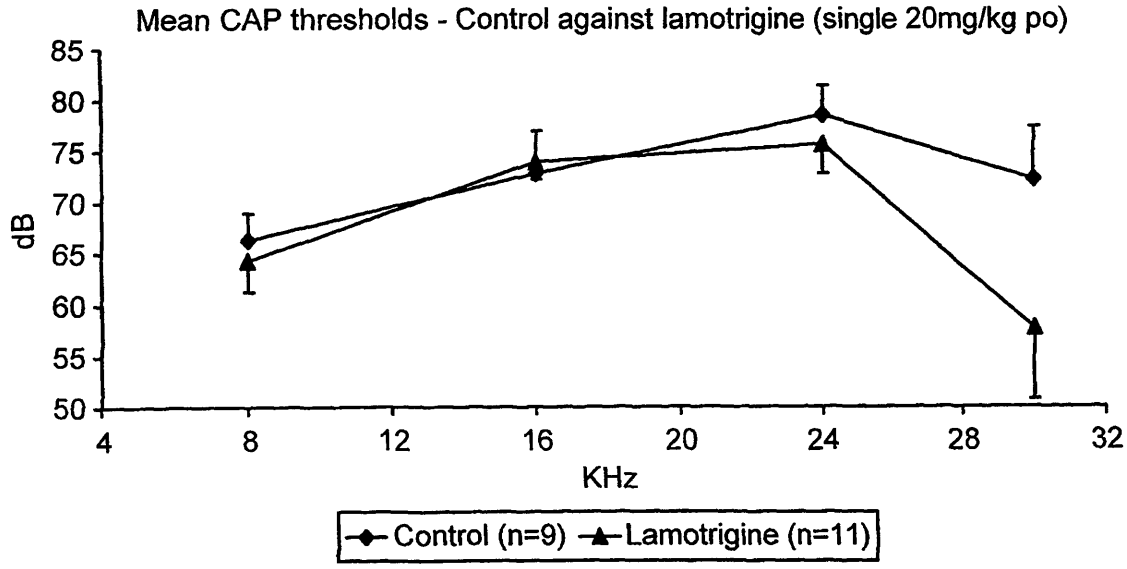


FIG. 3

The effect of LTG on CAP threshold shifts after exposure to noise (110dB SPL)

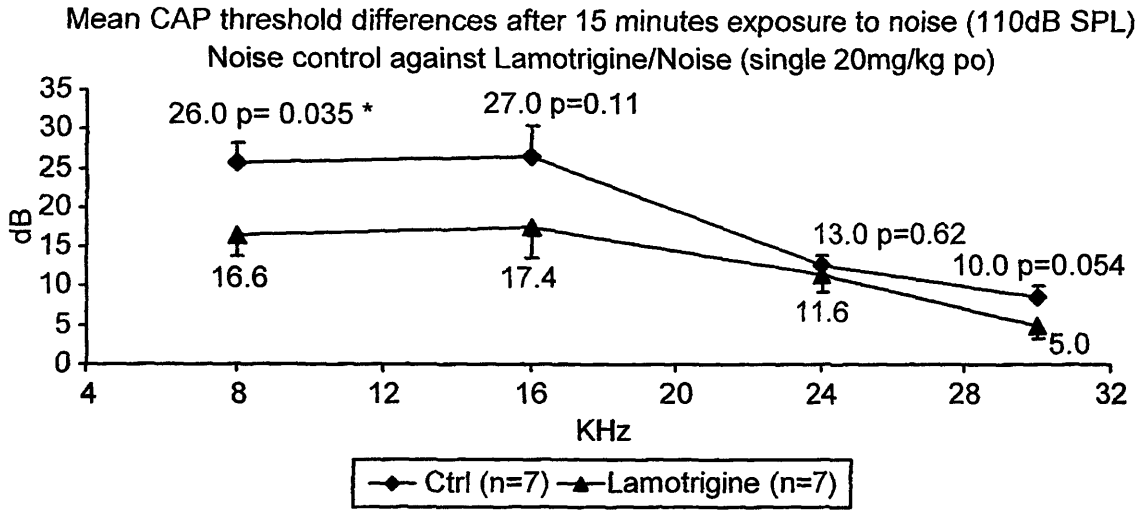
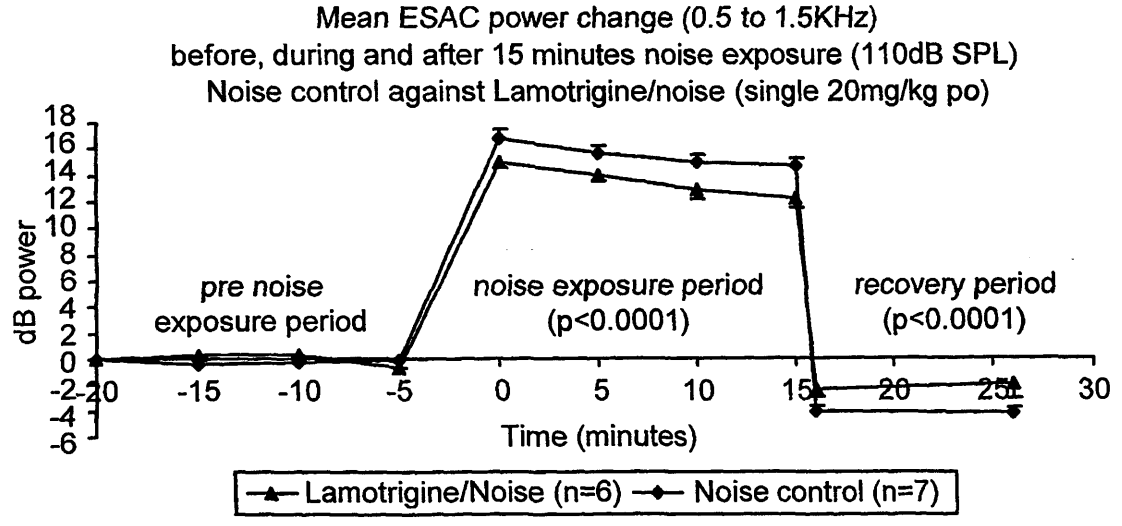


FIG. 4

The effect of LTG on ESAC before during and after noise exposure (110dB spl)



INTERNATIONAL SEARCH REPORT

Int ›nal Application No
PCT/EP 01/11498

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, CHEM ABS Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIMPSON, J. J. ET AL.: "The Assessment of Lamotrigine, an Antiepileptic Drug, in the Treatment of Tinnitus" THE AMERICAN JOURNAL OF OTOLOGY, vol. 20, September 1999 (1999-09), pages 627-631, XP008000630 the whole document	1-6

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2002	27/02/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Beyss, E

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No. PCT/EP 01 /11498

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1, 2, 5, 6

Present claims 1, 2, 5 ,6 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compound lamotrigine.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

REFERENCES

- Ades HW and Engstrom H (1976). Anatomy of the Inner Ear. *In*; Handbook of Sensory Physiology. Eds. Keidel WD and Neff WD, **5(1)** 125-158.

- Adelman WJ and Palti Y (1969). The effects of external potassium and long duration of voltage conditioning on the amplitude of sodium currents in the giant axon of the squid, *Loligo pealei*. Journal of general physiology, **54**, 589-606.

- d'Aldin C, Eybalin M, Puel JL, Charachon G, Ladrech S, Renard N and Pujol R (1995). Synaptic connections and putative functions of the dopaminergic innervation of the guinea pig cochlea. European Archives of Oto-Rhino-Laryngology **252**, 270-274.

- d'Aldin C, Puel JL, Leducq R, Crambes O, Eybalin M. and Pujol R (1995/97). Effects of a dopaminergic agonist in the guinea pig cochlea. Hearing Research, **90**, 202-211 (Erratum appears in Hearing Research, 1997, **103[1-2]** 225).

- Altschuler R, Miller J. Raphael Y and Schacht, J. (1999). Strategies for protection of the inner ear from NIHL. *In*; Cochlear pharmacology and Noise Trauma. Eds. Prasher D and Canlon B. NRN publications, London, 98-112.

- Ashmore JF (1987). A fast motile response in guinea pig OHCs: the cellular basis of the cochlear amplifier. Journal of Physiology, **388**, 323-347.

- Attias J, Sohmer H, Gold S, Haran I and Shahar A (1990). Noise and hypoxia induced temporary threshold shifts in rats studied by ABR. Hearing Research, **45(3)** 247-52.

- Axelsson A. (1968). The vascular anatomy of the cochlea in the guinea pig and man. Acta Otolaryngologica (Supplement), **243**, 1-134.

- Barlow HB and Mollon JD (1982). The senses. Cambridge University Press.

- Beagley HA (1979). Auditory Investigations: The scientific and technological basis. OUP. Oxford.

- Berg-Johnsen J and Langmoen IA (1986). Isoflurane effects in rat hippocampal cortex: a quantitative evaluation of different cellular sites of action. Acta Physiologica Scandinavica, **128(4)** 613-8.

- Billett TE, Thorne, PR and Gavin JB (1989). The nature and progression of injury in the organ of Corti during ischemia. Hearing Research, **41**, 189-198.

- Boettcher FA, Spongr VP and Salvi RJ (1992). Physiological and histological changes associated with the reduction in threshold shift during interrupted noise exposure. Hearing Research, **62(2)** 217-36.

- Brown MC, Nuttall AL and Masta RI (1983). Intracellular recordings from cochlear inner hair cells: effects of stimulation of the crossed olivocochlear efferents. Science, **222 (4619)** 69-72.

- Brownell WE, Bader CR, Bertrand D and Ribaupierre Y (1985). Evoked mechanical responses of isolated cochlear OHCs. Science, **227**, 194-196.

- Bruel and Kjaer Product Data Bulletin (1998). Noise and Vibration Analysis - Type 7700. Base Software for PULSE, the Multi-analyzer System Version **BP1795-14**.

- Brummett, R and Fox, K.E (1982). Studies of aminoglycoside ototoxicity in animal models. *In*; The aminoglycosides. Microbiology, Clinical Use and Toxicology. Eds: Whelton A and Ness HC. New York, Marcel Dekker, 419-452.

- Bruno V, Scapagnini U and Canonico PL (1993). Excitatory amino acids and neurotoxicity. Functional Neurology, **8**, (4), 279-292.

- Cannell H (1972). Pentobarbitone sodium anaesthesia for oral and immunological procedures in the guinea-pig. Laboratory Animals, **6(1)** 55-60.

- Carter A (1998). The importance of voltage-dependent sodium channels in cerebral ischaemia. Amino Acids, **14**, 159–169.

- Catterall, WA (1992). Cellular and molecular biology of voltage-gated sodium channels. Pharmacological Reviews **72** (4 - supplement) s15 - s48.

- Catterall, WA (1993a). Structure and modulation of Na^+ and Ca^{2+} channels. Annals New York Academy of Sciences, **707**, 1-19.

- Catterall, WA (1993b). Structure and function of voltage gated ion channels. Trends in Neurological Sciences, **16 (12)** 500-506.

- Catterall, WA (1994). Molecular mechanisms of inactivation and modulation of sodium channels, Renal Physiology & Biochemistry, **17**, 121-125.

- Catterall WA (2000). Structure and regulation of voltage-gated Ca^{2+} channels. Annual Reviews of Cell and Developmental Biology, **16**, 521-555.

- Cazals Y and Huang ZW (1996). Average spectrum of cochlear activity: a possible synchronized firing, its olivo-cochlear feedback and alterations under anaesthesia. Hearing Research, **101**, 81-92.

- Cazals Y, Horner KC and Huang ZW (1998). Alterations in average spectrum of cochleoneural activity by long-term salicylate treatment in the guinea pig: a plausible index of tinnitus. Journal of Neurophysiology, **80**, 2113-2120.

- Cazals Y (2000). Auditory sensori-neural alterations induced by salicylate. Progress in Neurobiology, **62 (6)** 583-631.

- Chen GD, McWilliams ML and Fechter LD (1999). Intermittent noise-induced hearing loss and the influence of carbon monoxide. Hearing Research, **138(1-2)** 181-91.

- Chen GD, Kong J, Reinhard K and Fechter LD (2001). NMDA receptor blockage protects against permanent noise-induced hearing loss but not its potentiation by carbon monoxide. Hearing Research, **154(1-2)** 108-15.

- Chiodo AA and Alberti PW (1994). Experimental, clinical and preventive aspects of ototoxicity. European Archives of Otorhinolaryngology, **251,** 7, 375-92.

- Choi DW and Rothman SM (1990). The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annual Review in Neuroscience, **13**, 171-182.

- Clare, JJ, Tate SN, Nobbs M and Romanos MA (2000). Voltage-gated sodium channels as therapeutic targets. Drug development today, **5 (11)**, 506-520.

- Clark WW (1991). Recent studies of temporary threshold shift (TTS) and permanent threshold shift (PTS) in animals. Journal of the Acoustical Society of America, **90** (1), 155-163.

- Cody AR and Russell IJ (1985). Outer hair cells in the mammalian cochlea and noise-induced hearing loss. Nature, **315,** 662-5.

- Cody AR and Russell IJ (1987). The response of hair cells in the basal turn of the guinea-pig cochlea to tones. Journal of Physiology, **383**, 551-569.

- Cody AR and Russell IJ (1988). Acoustically overstimulated hearing loss: Intracellular studies in the guinea pig cochlea. Hearing Research. **35**, 59-70.

- Comis SD (1970). Centrifugal inhibitory processes affecting neurones in the cat cochlear nucleus. Journal of Physiology, **210**, 751-60.

- Conlee JW, Abdul-Baqi KJ, McCandless GA and Creel DJ (1986). Differential susceptibility to noise-induced permanent threshold shift between albino and pigmented guinea pigs. Hearing Research, **23(1)** 81-91.

- Cooper NP (1989). On the peripheral coding of complex auditory stimuli: Temporal discharge patterns in guinea-pig cochlear nerve fibres. PhD Thesis, Keele University.

- Cycowicz Y, Schmuel M, Freeman S, Anszelbaum A and Sohmer H (1988). Perinatal hypoxia and auditory brainstem response thresholds: No evidence of permanent hearing loss. Hearing Research, **33**, 239-244.

- Davis AC (1983). Hearing disorders in the population; first phase findings of the MRC National Study of Hearing. *In*; Hearing science and hearing disorders. Eds. Lutman ME and Haggard MP. New York, Academic Press. **35.**

- Davis H (1958). Transmission and transduction in the cochlea. Laryngoscope, **68**, 359-382.

- Dawson R jr, Beal F, Bondy SC, Di Monte DA and Isom GE (1995). Excitotoxins, ageing and environmental neurotoxins: Implications for understanding human neurodegenerative diseases. Toxicology and Applied Pharmacology, **134**, 1-17.

- Dolan DF, Xi L and Nuttall AL (1989). Characterization of an EPSP-like potential recorded remotely from the round window. Journal of the Acoustical Society of America, **86**, 2167-2171.

- Dolan DF, Nuttall, AL and Avinash G (1990). Asynchronous neural activity recorded from the round window. Journal of the Acoustical Society of America, **87**, 2621-2627.

- Dugan LL and Choi DW (1994). Excitotoxicity, free radicals and cell membrane changes. Annals of Neurology, **35**, (suppl.) S17-21.

- Eaholtz G, Scheuer T and Catterall AW (1994). Restoration of inactivation and block of open sodium channels by an inactive gate peptide. Neuron, **12**, 1041-1048.

- Ertel EA, Campbell KP, Harpold MM, Hofmann F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW and Catterall WA (2000). Nomenclature of Voltage-Gated Calcium Channels. Neuron, **25**, 533–535 2000.

- Evans EF (1972). The frequency response and other properties of single fibres of the guinea pig cochlear nerve. Journal of physiology, **226**, 263-287.

- Evans EF and Nelson PG (1973). The responses of single neurones in the cochlear nucleus of the cat as a function of their location and the anaesthetic state. Experimental Brain Research, **17(4)**, 402-27.

- Evans EF (1974). The effects of hypoxia on the tuning of single cochlear nerve fibres. Journal of physiology, **238**, 65-67.

- Evans EF (1975). The cochlear nerve and cochlear nucleus. *In*; Handbook of sensory physiology, **2**: Auditory Systems. Eds. Keidel WD and Neff WD, Springer Verlag, Berlin, 1-108.

- Evans EF and Wilson JP (1975). Cochlear tuning properties: concurrent basilar membrane and single nerve fibre measurements. Science, **190**, 1218-1221.

- Evans EF, Wilson JP and Borerwe TA (1981). Animal models of tinnitus. CIBA Foundation Symposium, **85**, 108-38.

- Eybalin M, Calas A and Pujol R (1983). Radioautographic study of the symphathetic fibers in the cochlea. Acta Otolaryngoligica, **96**, 69-74.

- Fechter LD, Thorne PR and Nuttall AL (1987) Effects of carbon monoxide on cochlear electropyhsiology and blood flow. Hearing Research, **27**, 37-45.

- Fechter LD, Young JS and Carlisle L (1988). Potentiation of noise induced threshold shifts and hair cell loss by carbon monoxide. Hearing Research, **34**, 39-48.

- Fechter LD, Chen GD, Rao D and Larabee, J (2000). Predicting Exposure Conditions that Facilitate the Potentiation of Noise-Induced Hearing Loss by Carbon Monoxide. Toxicological Sciences, **58**, 315-323.

- Fern R, Ransom BR, Stys PK and Waxman SG (1993). Pharmacological protection of CNS white matter during anoxia: actions of phenytoin, carbamazepine and diazepam. Journal of Pharmacology & Experimental Therapeutics, **266(3)**, 1549-55.

- Fernandez C (1952). Dimensions of the cochlea in the guinea pig. Journal of the Acoustical Society of America, **24**, 519-523.

- Flecknell PA and Mitchell M (1984). Midazolam and fentanyl-fluanisone: assessment of anaesthetic effects in laboratory rodents and rabbits. Laboratory Animals, **18(2)** 143-6.

- Furness DN, Hackney CM and Steyger PS (1990). The organisation of microtubules in cochlear hair cells. J. Electr. Mic. Tec., **15**, 261-279.

- Gafni M and Sohmer H (1976). Intermediate endocochlear potential levels induced by hypoxia. Acta Otolaryngol, **82**, 354-358.

- Gillespie PG (1995). Molecular machinery of auditory and vestibular transduction. Current Opinion in Neurobiology, **5**, 449-455.

- Gil Loyzaga P and Pares-Herbute N (1989). HPLC detection of DA and NA in the cochlea of adult and developing rats. Developmental Brain Research, **48**, 157-160.

- GlaxoSmithkline Internal Report SR1998/0004/00 (1998). Update on the modulatory effects of lamotrigine on recombinant and native voltage-gated ion channels.

- Godfraind T and Govoni S (1995). Recent advances in the pharmacology of Ca^{2+} and K^{+} channels. Trends in Pharmacological Sciences, **16(1)** 1-4.

- Goycoolea MV and Lundman L (1997). Round window membrane. Structure function and permeability: A review. Microscopy Research and Technique, **36**, 201-211.

- Graham SH, Chen J, Sharp FR and Simon RP (1993). Limiting ischemic injury by inhibition of excitatory amino acid release. Journal of Cerebral Blood Flow & Metabolism, **13(1)** 88-97.

- Graham SH, Chen J, Lan J, Leach MJ and Simon RP (1994). Neuroprotective effects of a use-dependent blocker of voltage-dependent sodium channels, BW619C89, in rat middle cerebral artery occlusion. Journal of Pharmacology and Experimental Therapeutics, **269**, 854–859.

- Green CJ (1975). Neuroleptanalgesic drug combinations in the anaesthetic management of small laboratory animals. Laboratory Animals, **9(3)** 161-78.

- Guyton AC and Hall JE (1996). Textbook of Medical Physiology. WB Saunders Co, 9th edition.

- Hainsworth AH, Spadoni F, Lavaroni F, Bernardi G and Stefani A (2001). Effects of extracellular pH on the interaction of sipatrigine and lamotrigine with high-voltage-activated (HVA) calcium channels in dissociated neurones of rat cortex. Neuropharmacology, **40**, 784-791.

- Hakuba N, Koga K, Gyo K, Usami SI and Tanaka K (2000). Exacerbation of noise-induced hearing loss in mice lacking the glutamate transporter GLAST. The Journal of Neuroscience, **20(23)**, 8750-8753.

- Hamel Volunteer Fire Department, Minnesota (1995). Internet Datasource.

- Hamernick RP and Henderson D (1976). The potentiation of noise by other ototraumatic agents. *In;* Effects of noise on hearing. Eds. Henderson D, Hamerick RP, Dosanjh DS and Mills JH. Raven Press, 291-307.

- Hamernick RP, Henderson D and Salvi RJ (1980). Contribution of animal studies to our understanding of impulse noise induced hearing loss. Scandinavian Audiology Suppl. **12**, 128-146.

- Harkness JE, Wagner JE (1989). The Biology and Medicine of Rabbits and Rodents; 3rd Edition, Lea and Febiger, Philadelphia.

- Haupt H, Scheibe F and Ludwig C (1993). Changes in cochlear oxygenation, microcirculation and auditory function during prolonged general hypoxia. European Archives of Otorhinolaryngology, **250(7)**, 396-400.

- Heinemann SH, Terlau H, Stuhmer W, Imoto K and Numa S (1992). Calcium channel characteristics conferred on the sodium channel by single mutations. Nature, **356 (6368)** 441–443.

- Heffner RS, Heffner, EH and Masterton B (1971). Behavioural measurements of absolute and frequency difference mechanisms in the guinea pig. Journal of the Acoustical Society of America, **49(6)** 1888-1895.

- Henderson D, Hamernik RP and Sitler RW (1974). Audiometric and histological correlates of exposure to 1-msec noise impulses in the chinchilla. Journal of the Acoustical Society of America, **56,** 1210-1221.

- Henderson D, Hynson K and Hamernik RP (1977). Importance of the wave form of the impulse in formulating an impulse noise damage risk criteria. Journal of the Acoustical Society of America, **62, Suppl. 4**.

- Henderson D, Hamernik RP, Salvi RJ and Ahroon WA (1983). Comparison of auditory-evoked potentials and behavioral thresholds in the normal and noise-exposed chinchilla. Audiology, **22(2)** 172-80.

- Henderson D and Hamernick RP (1995). Biologic bases of noise-induced hearing loss. Occupational Medicine, **10(3)** 513-34.

- Henderson D and Hamernick RP (1996). Impulse noise: Critical review. Journal of the Acoustical Society of America, **65,** 1238-1248.

- Henderson D, McFadden SL, Zheng XY, Kopke R and Hight N (1999). Intervention possibilities for NIHL. *In*; Cochlear pharmacology and Noise Trauma. Eds. Prasher D and Canlon B, NRN Press, London, 85-97.

- Hirokawa N and Tilney LG (1982). Interactions between actin filaments and between actin filaments and membranes in quick-frozen and deeply etched hair cells of the chick ear. Journal of Cell Biology, **95**, 249-61.

- Hodgkin AL and Huxley AF (1952a). Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. Journal of Physiology of London, **116**, 449-472.

- Hodgkin AL and Huxley AF (1952b). The components of membrane conductance in the giant axon of *Loligo*. Journal of Physiology of London, **116**, 473-496.

- Hodgkin AL and Huxley AF (1952c). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. Journal of Physiology of London, **116**, 497-506.

- Holley MC and Ashmore J (1988). A cytoskeletal spring in cochlear OHCs. Nature, **353**, 635-637.

- Hu BH, Zheng XY, McFadden SL, Kopke RD and Henderson D (1997). R-phenylisopropyladenosine attenuates noise-induced hearing loss in the chinchilla. Hearing Research, **113(1-2)** 198-206.

- Hudspeth AJ (1985). The cellular basis of hearing: the biophysics of hair cells. Science, **230**, 745-752.

- Hyodo J, Hakuba N, Koga K, Watanabe F, Shudou M, Taniguchi M and Gyo K (2001). Hypothermia reduces glutamate efflux in perilymph following transient cochlear ischemia. NeuroReport, **12(9)** 1983-7.

- Inoue M, Ates N, Vossen JM and Coenen AM (1994). Effects of the neuroleptanalgesic fentanyl-fluanisone (Hypnorm) on spike-wave discharges in epileptic rats. Pharmacology, Biochemistry & Behaviour, **48(2)** 547-51.

- Johnstone BM, Patuzzi R and Yates GK (1986). Basilar membrane measurements and the travelling wave. Hearing Research, **22**, 147-53.

- Jorgensen M, Tygesen CK and Andersen PH (1995). Ionotropic glutamate receptors - Focus on Non-NMDA receptors. Pharmacology and Toxicology, **76**, 312-319.

- Kawaguchi K and Graham S (1997). Neuroprotective effects of the glutamate release inhibitor 619C89 in temporary middle cerebral artery occlusion. Brain Research, **749**, 131–134.

- Kawase T and Liberman MC (1993). Antimasking effects of the olivocochlear reflex. I. Enhancement of compound action potentials to masked tones. Journal of Neurophysiology, **70(6)** 2519-32.

- Kemp DT (1978). Stimulated acoustic emissions from within the human auditory system. Journal of the Acoustical Society of America, **64**, 1386-1391.

- Khanna SM and Leonard DGB (1982). Basilar membrane tuning in the cat cochlea. Science, **215**, 305-306.

- Kiang NYS, Watanabe T, Thomas EC and Clark LF (1965). Discharge patterns of single fibres in the cat's auditory nerve. Res Monogr, 35, MIT Press, Cambridge.

- Kiang NYS, Liberman MC and Levine RA (1976). Auditory-nerve activity in cats exposed to ototoxic drugs and high-intensity sounds. Annals of Otology, Rhinology & Laryngology, **85**, 752-768.

- Kiang NY, Liberman MC, Sewell WF and Guinan JJ (1986). Single unit clues to cochlear mechanisms. Hearing Research, **22**, 171-82.

- Kiedrowski L, Brooker G, Costa E and Wroblewski JT (1994a). Glutamate impairs neuronal calcium extrusion while reducing sodium gradient. Neuron, **12(2)** 295-300.

- Kiedrowski L, Wroblewski JT and Costa E (1994b). Intracellular sodium concentration in cultured cerebellar granule cells challenged with glutamate. Molecular Pharmacology, **45(5)** 1050-1054.

- Konishi T, Butler RA and Fernandez C (1961). Effect of anoxia on cochlear potentials. The Journal of the Acoustical Society of America, **33 (3)** 349-356.

- Kossl M and Russell IJ (1992). The phase and magnitude of hair cell receptor potentials and frequency tuning in the guinea pig cochlea. The Journal of Neuroscience, **12(5)** 1575-1586.

- Krnjevic K (1992). Cellular and synaptic actions of general anaesthetics. General Pharmacology, **23(6)** 965-75.

- Kros C and Crawford AC (1988). Components of the membrane current in guinea pig hair cells. In: Cochlear mechanisms. Eds. Wilson JP and Kemp DT. Plenum press, New York, 189-197.

- Kuijpers W and Bonting SL (1969). Studies on the Na^+ -K^+ activated ATPase. XXIV. Localization and properties of ATPase in the inner ear of the guinea pig. Biochimica et Biophysica Acta, **173(3)** 477-85.

- Kuijpers W and Bonting SL (1970). The cochlear potentials. I. The effect of ouabain on the cochlear potentials of the guinea pig. Pflugers Archive - European Journal of Physiology, **320(4)** 348-58.

- Langmoen IA, Larsen M and Berg-Johnsen J (1995). Volatile anaesthetics: cellular mechanisms of action. European Journal of Anaesthesiology, **12(1)** 51-8.

- Langosch JM, Zhou X-J, Frick A, Grunze H and Walden J (2000). Effects of lamotrigine on field potentials and long term potentiation in guinea pig hippocampal slices. Epilepsia, **41(9)** 1102-1106.

- Larsen M, Grondahl TO, Haugstad TS and Langmoen IA (1994). The effect of the volatile anesthetic isoflurane on Ca^{2+} dependent glutamate release from rat cerebral cortex. Brain Research, **663(2)** 335-7.

- Larsen M, Hegstad E, Berg-Johnsen J and Langmoen IA (1997). Isoflurane increases the uptake of glutamate in synaptosomes from rat cerebral cortex. British Journal of Anaesthesia, **78(1)** 55-9.
- Larsen M and Langmoen IA (1998). The effect of volatile anaesthetics on synaptic release and uptake of glutamate. Toxicology Letters, **100-101**, 59-64.

- Leach M (1993). BW619C89, a glutamate release inhibitor, protects against focal cerebral ischemic damage. Stroke, **24**, 1063–1067.

- Leach MJ, Lees G and Riddall DR (1995). Lamotrigine: Mechanisms of action. *In;* Antiepileptic Drugs, 4th Edition. Eds. Mattison RH and Meldrum BS. Raven Press, New York. 861 – 869.

- Lees G and Leach MJ (1993). Studies on the mechanism of action of the novel anticonvulsant lamotrigine (Lamictal) using primary neuroglial cultures from rat cortex. Brain research, **612**, 190.

- Liberman MC and Kiang NYS. (1978). Acoustic trauma in cats: cochlear pathology and auditory nerve activity. Acta Otolaryngologica, suppl. 358, *In*; Hearing (1987). Eds. Haggard MP and Evans EF. British Medical Bulletin, 43(4), Churchill Livingstone.

- Liberman MC (1982). Single-neuron labelling in the cat auditory nerve. Science, **216**, 1239-1241. *In* : Hearing (1987), British Medical Bulletin, 43, (4). Eds. Haggard, MP and Evans, E.F. Churchill Livingstone.

- Liberman MC and Dodds LW (1987). Acute ultrastructural changes in acoustic trauma: serial-section reconstruction of stereocilia and cuticular plates. Hearing Research, **26(1)** 45-64.

- Liberman MC (1984). Single-neuron labeling and chronic cochlear pathology. I. Threshold shift and characteristic-frequency shift. Hearing Research, **16(1)** 33-41.

- Liberman MC and Dodds LW (1984a). Single-neuron labelling and chronic cochlear pathology. II. Stereocilia damage and alterations of spontaneous discharge rates. Hearing Research, **16(1)** 43-53.

- Liberman MC and Dodds LW (1984b). Single-neuron labelling and chronic cochlear pathology. III. Stereocilia damage and alterations of threshold tuning curves. Hearing Research, **16(1)** 55-74.

- Liberman MC and Kiang NY (1984c). Single-neuron labelling and chronic cochlear pathology. IV. Stereocilia damage and alterations in rate and phase-level functions. Hearing Research, **16(1)** 75-90.

- Liberman MC (1987). Chronic ultrastructural changes in acoustic trauma: serial-section reconstruction of stereocilia and cuticular plates. Hearing Research, **26(1)** 65-88.

- Liberman MC (1991). The olivocochlear efferent bundle and susceptibility of the inner ear to acoustic injury. Journal of Neurophysiology, **65(1)** 123-32.

- Lim DJ (1986). Functional structure of the Organ of Corti: A review. Hearing Research, **22**, 171-45.

- Liu Y and Fechter LD (1995). MK-801 Protects against Carbon Monoxide-Induced Hearing Loss. Toxicology and Applied Pharmacology, **132(2)** 196-202.

- Liu G, Scheuer T and Catterall AW (1998). Two residues in segment IVS6 of the brain type II Na^+ channel α subunit form part of the receptor site for lamotrigine. Journal of Biophysiology, **74**, 399.

- Lima da Costa D, Erre JP, Charlet de Sauvage R, Popelar J and Aran JM (1997). Bioelectrical cochlear noise and its contralateral suppression: relation to background activity of the eighth nerve and effects of sedation and anesthesia. Experimental Brain Research, **116,** 259-69.

- Lustig HS, von Brauchitsch KL, Chan J and Greenberg DA (1992). A novel inhibitor of glutamate release reduces excitotoxic injury *in vitro*. Neuroscience Letters, **143(1-2)** 229-32.

- Manley GA. Robertson D (1976). Analysis of spontaneous activity of auditory neurones in the spiral ganglion of the guinea-pig cochlea. Journal of Physiology, **258(2)** 323-36

- Marban E, Yamagashi T and Tomaselli GF (1998). Structure and function of voltage-gated sodium channels. Journal of physiology, **508, 3**, 647-657.

- Martin WH (1995). Spectral analysis of brain activity in the study of tinnitus. *In*; Mechanisms of Tinnitus. Eds. Vernon JA and Møller AR. Allyn and Bacon, Boston, MA, 163-180.

- Martin, WH, Schwegler JW, Shi Y, Pratt H and Adler S (1996). Developing an objective measurement tool for evaluating tinnitus: spectral averaging. *In*: Proceedings of 5th International Tinnitus Seminar, Eds. Reich GE and Vernon JA. American Tinnitus Association, Portland, Oregon, 127-133.

- Mayer ML (1987). Cellular mechanisms underlying excitotoxicity. Trends in Neuroscience, **10**, 59-61.

- McPhee JC, Ragsdale DS, Scheuer T and Catterall WA (1994). A mutation in segment IVS6 disrupts fast inactivation of sodium channels. Proceedings of the National Academy of Sciences of the United States of America, **91(25)** 12346-50.

- McPhee JC, Ragsdale DS, Scheuer T and Catterall WA (1995). A critical role for transmembrane segment IVS6 of the sodium channel alpha subunit in fast inactivation. Journal of Biological Chemistry, **270(20)** 12025-34.

- Meade ML and Dillon CR.(1991). Signals and systems: Models and behaviour, Wokingham : Van Nostrand Reinhold.

- Mertens J and Muller-Deile J (1991). The value of fentanyl/diazepam anaesthesia for experimental operations and recordings of compound action potentials in the guinea pig cochlea. European Archives of Oto-Rhino-Laryngology **248**, 402-405.

- Miller JD (1974). Effects of noise on people. Journal of the acoustical society of America, **56**, 729-64.

- Miller JJ (1985). *In*; Handbook of Ototoxicity, 1st edition, CRC Press, Inc.

- Mills JH, Gilber RM and Adkins WY (1979). Temporary threshold shifts in humans exposed to octave band noise for 16-24 hours. Journal of the Acoustical Society of America, **65**, 1238-1248.

- Mills JH and Going JA (1982). Review of environmental factors affecting hearing. Environmental Health Perspectives, **44**, 119-27.

- Mizobe T and Maze M (1995). Alpha 2-adrenoceptor agonists and anesthesia. International Anaesthesiology Clinics, **33(1)** 81-102.

- Mnich Z (1971). Permeability of Reissner's Membrane in the isolated ear of the guinea pig. Acta Otolaryngology, **71**, 27-33.

- Monsell, EM et al (1997). Non hereditary hearing loss. *In*: Clinical Otology. Eds. Hughes GB and Pensak ML, Thieme. New York, 289-312.

- Morest, DK (1982). Degeneration in the brain following exposure to noise. *In*: New Perspectives on Noise- induced Hearing Loss. Eds. Hamernik RP and Henderson D. Raven press, New York, 87-93.

- Mountain DC (1986). Electrochemical properties of hair cells. *In*: Neurobiology of Hearing. Eds. Altschuler RA, Hoffman DW and Bobbins RP. Raven Press, New York, 77-90.

- Mountain DC and Cody AR (1999). Multiple modes of inner hair cell stimulation. Hearing Research, **132(1-2)** 1-14.

- Mulheran M and Evans EF (1988). The dependence of the ARP of nerve fibres in the guinea pig on arterial blood pressure. British Journal of Audiology, **21**, 309-310.

- Mulheran M. (1990). The effects of systemic blood pressure, salicylate and quinine on the activity of the guinea pig auditory nerve. PhD Thesis, University of Keele.

- Nakagawa T, Komune S, Uemura T, Akaike N (1991). Excitatory amino acid response in isolated spiral ganglion cells of guinea pig cochlea. Journal of Neurophysiology, **65(3)** 715-23.

- Neely ST and Kim DO (1983). An active cochlear model showing sharp tuning and high sensitivity. Hearing Research, **9**, 123-131.

- Neely ST and Kim DO (1986). A model for active elements in cochlear biomechanics. Journal of the Acoustical Society of America, **79**, 1472-1480.

- Neugebauer DC, Thurm U (1984). Chemical dissection of stereovilli from fish inner ear reveals differences from intestinal microvilli. Journal of Neurocytology, **13**, 797-808.

- Nowycky MC, Fox AP and Tsien RW (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. Nature, **316(6027)** 440-3.

- Nuttall AL and Lawrence M (1980). Endocochlear potentials and scala media oxygen tension during partial anoxia. American Journal of Otolaryngology, **1(2)** 147-153.

- Ohinata Y, Yamasoba T, Schacht J and Miller JM (2000). Glutathione limits noise-induced hearing loss. Hearing Research, **146(1-2)** 28-34.

- Palmer AR (1987). Physiology of the cochlear nerve and cochlear nucleus. British Medical Bulletin, **43(4)** 838-855.

- Palmer AM and Cater N (2001). The role of sodium channels on disease. Drug News Perspective, **14(9)** 568-576.

- Parsons DN, Dickins M and Morley TJ (1995). Lamotrigine: Absorption, distribution, and excretion *in* Antiepileptic drugs. 4th Edition, Raven Press, New York, 877-881.

- Parks TN (2000). The AMPA receptors of auditory neurons. Hearing Research, **147 (1-2)** 77-91.

- Patuzzi R and Robertson D (1988). Tuning in the mammalian cochlea. Physiological Reviews, **68(4)** 1009-82.

- Pickles JO (1985). Recent advances in cochlear physiology. Progress in Neurobiology, **24(1)** 1-42.

- Pickles JO (1991). An Introduction to the Physiology of Hearing, 2nd edition, Academic Press.

- Pieri L, Schaffner R, Scherschlicht R, Polc P, Sepinwall J, Davidson A, Mohler H, Cumin R, Da Prada M, Burkard WP, Keller HH, Muller RK, Gerold M, Pieri M, Cook L and Haefely W (1981). Pharmacology of midazolam, Arzneimittel-Forschung, **31(12a)** 2180-201.

- Pisani F, Pedale S, Macaione V, Torre V, Oteri G and Avanzini G (2001). Neuroprotective effects of lamotrigine and remacemide on excitotoxicity induced by glutamate agonists in isolated chick retina. Experimental Neurology, **170**, 162-170.

- Poole T (1999). The care and management of Laboratory animals. *In:* UFAW Handbook, 7th edition Blackwell Science 1999 Ed Oxford Chapter 23, 367-388.

- Prasher D (1999). New strategies for the prevention and treatment of noise induced hearing loss. *In* Cochlear Pharmacology and noise trauma. Eds. Prasher D and Canlon B. NRN publications, London.

- Puel JL, Bobbin RP and Fallon M (1988). The active process is affected first by intense sound exposure. Hearing Research, **37**, 53-64.

- Puel JL, Ladrech S, Chabert R, Pujol R and Eybalin M (1991). Electrophysiological evidence for the presence of NMDA receptors in the guinea pig cochlea. Hearing Research, **51**, 255-264.

- Puel JL, Pujol R, Tribillac F, Ladrech S and Eybalin M. (1994). Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. Journal of Comparative Neurology, **341**, 241-256.

- Puel JL (1995). Chemical synaptic transmission in the cochlea. Progress in Neurobiology, **47**, 449-476.

- Puel JL, Saffiedine S, D'Aldin CG, Eybalin M and Pujol R (1995). Synaptic regeneration and functional recovery after excitotoxic injury in the guinea pig cochlea. Comptes Rendus de l'Academie des Sciences - Serie III, Sciences de la Vie, **318 (1)** 67-75.

- Puel JL, Ruel J, d'Aldin CG and Pujol R (1998). Excitotoxicity and repair of cochlear synapses after noise-trauma induced hearing loss. NeuroReport, **9(9)** 2109-2114.

- Puel JL, dA'Aldin C, Ruel J, Ladrech S and Pujol R. (1999). Perspectives in inner ear pharmacology and clinical applications *In*: Cochlear pharmacology and Noise Trauma. Eds. Prasher D, and Canlon B, NRN Press London, 1-8.

- Pujol R, Lenoir M, Robertson D, Eybalin M and Johnstone BM (1985). Kainic acid selectively alters auditory dendrites connected with cochlear inner hair cells. Hearing Research, **18**, 145-151.

- Pujol R, Rebillard, G, Puel JL, Lenoir M, Eybalin M. and Recasens M. (1990). Glutamate neurotoxicity in the cochlea: A possible consequence of ischaemic or anoxic conditions occurring in ageing. Implication of non-NMDA and NMDA receptors in cochlear ischemia. Acta Otolaryngol (Stockh) suppl, **476,** 32-36.

- Pujol R, Puel JL and Eybalin M (1992). Implication of non-NMDA and NMDA receptors in cochlear ischemia. NeuroReport, **3**, 299-302.

- Pujol R, Puel JL, D'Aldin CG and Eybalin M (1993). Pathophysiology of the glutaminergic synapses in the cochlea. Acta Otolaryngology (Stockholm), **113**, 330-334.

- Pujol R (1994). Lateral and medial efferents: a double neurochemical mechanism to protect and regulate inner and outer hair cell function in the cochlea. British Journal of Audiology, **28 (4-5)** 185-91.

- Pujol R and Puel JL (1999). Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings. Annals of the New York Academy of Sciences, **884**, 249-54.

- Quaranta A, Portalatini P and Henderson D (1998). Temporary and permanent threshold shift: an overview. Scandinavian Audiology. Supplementum, **48**, 75-86.

- Ragsdale D, McPhee JC, Scheuer T and Catterall WA (1994). Molecular determinants of state-dependent block of Na^+ channels by local anaesthetic. Science, **265**, 1724-1728.

- Ragsdale DS, McPhee JC, Scheuer T and Catterall WA (1996). Common molecular determinants of local anaesthetic, antiarrythmic and anticonvulsant block of voltage gated Na^+ channels by local anaesthetic. Proceedings of the National Academy of Sciences of the United States of America, **93**, 9270-9275.

- Rambeck B and Wolf P (1993). Lamotrigine clinical pharmacokinetics. Clinical Pharmacokinetics, **25(6)** 433-43.

- Ramsey ER, Pellock JM, Garnett WR, Sanchez RM, Valakas AM, Wargin WA, Lai AA, Hubbell J, Chern WH, Allsup T and Otto V (1991). Pharmacokinetics and safety of lamotrigine (Lamictal®) in patients with epilepsy. Epilepsy Research, **10**, 191-200.

- Rang HP and Dale MM (1991). Pharmacology. 2nd Edition, Churchill Livingstone.

- Rao D and Fechter LD (2000). Protective effects of phenyl-N-tert-butylnitrone on the potentiation of noise-induced hearing loss by carbon monoxide. Toxicology & Applied Pharmacology, **167(2)** 125-31.

- Remmel RP and Sinz MW (1991). A quaternary ammonium glucuronide is the major metabolite of Lamotrigine in guinea pigs. *In vitro* and *in vivo* studies. Drug Metabolism & Disposition, **19(3)** 630-6.

- Rhodes WS (1971). Observations of the vibrations of the basilar membrane in the squirrel monkeys using the Mossbaeur technique. Journal of the Acoustical Society of America, **49**, 1218-1231.

- Rhodes WS (1978). Some observations on cochlear mechanics. Journal of the Acoustical Society of America, **64**, 158-176.

- Richens A (1992). Pharmacokinetics of Lamotrigne. Clinical update on lamotrigine a novel antiepileptic agent. *In*: International clinical practice series, Ed; Richens A, Kent Wells Medical, 21-27.

- Robertson D (1983). Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hearing Research, **9(3)** 263-78

- Robertson, (1984). Horse radish peroxidase injection of physiologically characterised afferent and efferent neurones in the guinea pig spiral ganglion. Hearing Research, **15**, 113-122.
- Ruel J, Chen C, Pujol R, Bobbin RP and Puel JL (1999). AMPA-preferring glutamate receptors in cochlear physiology of adult guinea-pig. Journal of Physiology, **518**, 667-680.

- Ruel J, Bobbin RP, Vidal D, Pujol R and Puel JL (2000). The selective AMPA receptor antagonist GYKI 53784 blocks action potential generation and excitotoxicity in the guinea pig cochlea. Neuropharmacology, **39(11)** 1959-73.

- Ruel J, Nouvian R, d'Aldin CG. Pujol R, Eybalin M and Puel JL (2001). Dopamine inhibition of auditory nerve activity in the adult mammalian cochlea. European Journal of Neuroscience, **14**, 977-86.

- Ruben PC, Starkus JG, Rayner MD (1992). Steady-state availability of sodium channels. Biophysical Journal, **61**, 941-955.

- Russell IJ and Cowley EM (1983). The influence of transient asphyxia on receptor potentials in IHCs of the guinea pig cochlear. Hearing Research, **11**, 373-384.

- Russell IJ (1987). The physiology of the organ of Corti. British Medical Bulletin, **43(4)** 802-820.

- Russell IJ and Sellick (1978). Intracellular studies of hair cells in the mammalian cochlea. Journal of Physiology, **284**, 261-290.

- Ryan AF (1988). Circulation of the inner ear II. The relationship between metabolism and blood flow in the cochlea. *In*: physiology of the ear. Eds. Jahn AF and Santos-Saachi J. Raven Press, New York, 317-326.

- Salonen JS and Eloranta M (1990). Biotransformation of medetomidine in the rat, Xenobiotica, 1990, **20(5)** 471-80.

- Salt AN and Ma Y (2001). Quantification of solute entry into cochlear perilymph through the round window membrane. Hearing Research, **154**, 88-97.

- Salvi RJ, Hamernik RP and Henderson D (1979a). Auditory nerve activity and cochlear morphology after noise exposure. Archives of Oto-Rhino-Laryngology, **224(1-2)** 111-6.

- Salvi RJ, Henderson D and Hamernik RP (1979b). Single auditory nerve fiber and action potential latencies in normal and noise-treated chinchillas. Hearing Research, **1**, 237-251.

- Salvi RJ, Hamernik RP and Henderson D (1983). Response patterns of auditory nerve fibers during temporary threshold shift. Hearing Research, **10(1)** 37-67.

- Santi PA (1988). Cochlear microanatomy and ultrastructure. *In*: Physiology of the ear. Eds. Jahn AF and Santos-Saachi J. Raven Press, New York, 271-293,

- Schacht J (1982). Biochemical aspects of noise-induced hearing loss. *In*: New Perspectives on Noise- induced Hearing Loss, edited by Hamernik RP and Henderson D., Raven press. New York, 95-102.

- Schreiner CE and Snyder RL (1987). A physiological animal model of peripheral tinnitus. *In*: Third International Tinnitus Seminar, Editor; Feldmann H, Harsch, Karlsrhue, Germany, 100-106.

- Selvadurai D, Etheridge S, Jones P and Mulheran M (2000). Pharmacological protection of auditory function against noise and hypoxia with MK-801. Clinical Otology, **25**, 570-571.

- Shaw NA (1988). The auditory evoked potential in the rat - a review. Progress in Neurobiology, **31**, 19-45.

- Simpson JJ, Gilbert AM, Weiner GM and Davies EW (1999). The assessment of Lamotrigine, an antiepileptic drug, in the treatment of tinnitus. The American Journal of Otology, **20**, 629-631.

- Smith SE and Meldrum BS (1995). Cerebroprotective effect of lamotrigine after focal ischaemia in rats. Stroke, **26**, 117-121.

- Smith SE, Lekieffre D, Sowinski P and Meldrum BS (1993). Cerebroprotective effect of BW619C89 after focal or global cerebral ischaemia in the rat. NeuroReport, **4**, 1339–1342.

- Sohmer H, Freeman S and Schmuel M (1989). ABR threshold is a function of blood oxygen level. Hearing Research, **40**, 87-92.

- Spoendlin H (1978). The afferent innervation of the cochlea. *In* Evoked electrical activity in the auditory nervous system. Eds. Naunton RF and Fernandez C. Academic Press, London, 21-39.

- Stebbins WC, McGinn C, Feitosa A, Moody D, Prosen CA and Serafin JV (1981). Animal models in the study of ototoxic hearing loss. *In*: Aminoglycoside ototoxicity. Eds. Lerner SA, Matz GA and Hawkins JE. Boston. Little Brown & Co., 5-29.

- Stefani A, Spadoni F, Sinisalchi A and Bernadi G (1996). Lamotrigine inhibits calcium currents in cortical neurons: functional implications. European journal of pharmacology, **307**, 113-116.

- Stefani A, Spadoni F and Bernadi G (1997). Voltage activated calcium channels: Targets of antiepileptic drug therapy? Epilepsia, **38 (9)**, 959-965.

- Stühmer W, Conti, F, Suzuki, H, Wang, X, Noda, M, Yahagi, N, Kubo, H and Numa, S (1989). Structural parts involved in activation and inactivation of the sodium channel. Nature, **339**, 597-603.

- Stys PK, Ransom BR and Waxman SG (1992). Tertiary and quaternary local anaesthetics protect CNS white matter from anoxic injury at concentrations that do not block excitability. Journal of Neurophysiology, **67(1)** 236-40.

- Sullivan MJ and Conolly RB (1988). Dose-response hearing loss for white noise in the Sprague-Dawley rat. Fundamental and Applied Toxicology, **10**, 109-113.

- Sun F and Faden A (1995). Neuroprotective effects of 619C89, a use-dependent sodium channel blocker, in rat traumatic brain injury. Brain Research, **673**, 133–140.

- Szatkowski M and Attwell D (1994). Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends in Neurosciences, **17(9)** 359-65.

- Tasaki I (1954). Nerve impulses in individual auditory nerve fibers of guinea pig. Journal of Neurophysiology, **17**, 97-122.

- Tasker RC, Coyle JT and Vornov JJ (1992). The regional vulnerability to hypoglycemia-induced neurotoxicity in organotypic hippocampal culture: protection by early tetrodotoxin or delayed MK-801. Journal of Neuroscience, **12(11)** 4298-308.

- Taylor CP and Meldrum BS (1995). Na^+ channels as targets for neuroprotective drugs. Trends in Pharmacological Sciences, **16**, 309-315.

- Taylor CP, Burke SP and Weber ML (1995). Hippocampal slices: glutamate overflow and cellular damage from ischemia are reduced by sodium-channel blockade. Journal of Neuroscience Methods, **59(1)** 121-8.

- Taylor CP and Narasimhan LS. (1997) Sodium channels and therapy of central nervous system diseases. Advances in Pharmacology, **39**, 47–98

- The National Occupational Safety and Health Act of 1970. Public Law 91-596. 91st Congress, S. 2193.

- Thornton C, Creagh-Barry P, Jordan C, Luff NP, Dore CJ, Henley M and Newton DE (1992). Somatosensory and auditory evoked responses recorded simultaneously: differential effects of nitrous oxide and Isoflurane. British Journal of Anaesthesia, **68(5)** 508-14.

- Von Bekesy G (1960). Experiments in hearing. Wiley, New York.

- Von Wegerer J, Hesslinger B, Berger M and Walden J (1997). A calcium antagonistic effect of the new antiepileptic drug lamotrigine. European Neuropsychopharmacology, **7**, 3037-3040.

- Usami S, Matsubara A, Fujita S, Shinkawa H and Hayashi M (1995). NMDA (NMDAR1) and AMPA-type (GluR2/3) receptor subunits are expressed in the inner ear. NeuroReport, **6(8)** 1161-4.

- Wang SJ, Huang CC, Hsu KS, Tsai JJ and Gean PW (1996). Inhibition of N-type calcium currents by lamotrigine in rat amygdalar neurones. Neuroreport, **7**, 3037-40.

- Wang GK, Quan C and Wang S (1998). A common local anesthetic receptor for benzocaine and etidocaine in voltage-gated mu1 Na^+ channels. Pflugers Archive - European Journal of Physiology, **435(2)**, 293-302.

- Warr WB (1978). The olivocochlear bundle: its origins and terminations in the cat. *In*; Evoked Electrical Activity in the Auditory Nervous System. Eds. Naunton RF and Fenandez C. Academic Press, New York and London, 43-63.

- Watanabe F, Koga K, Hakuba N and Gyo K (2001). Hypothermia prevents hearing loss and progressive hair cell loss after transient cochlear ischemia in gerbils. Neuroscience, **102(3)** 639-45.

- Weber ML and Taylor CP (1994). Damage from oxygen and glucose deprivation in hippocampal slices is prevented by tetrodotoxin, lidocaine and phenytoin without blockade of action potentials. Brain Research, **664(1-2)** 167-77.

- West J, Patton D, Scheuer T, Wang Y, Goldin A, Catterall WA (1992). A cluster of hydrophobic amino acid residues required for fast Na^+ channel inactivation. Proceedings of the National Academy of Sciences of the USA, **89**, 10910-10914.

- Wiard RP, Dickerson MC, Beek O, Norton R and Cooper BR (1995). Neuroprotective properties of the novel antiepileptic lamotrigine in a gerbil model of global cerebral ischaemia. Stroke, **26(3)** 466-472.

- Wiederhold ML (1970). Variations in the effects of electric stimulation of the crossed olivocochlear bundle on cat single auditory-nerve-fiber responses to tone bursts. Journal of the Acoustical Society of America, **48(4)** 966-77.

- Wilson JP and Johnstone JR (1975). Basilar membrane and middle ear vibration in the guinea pig measured by capacitive probe. Journal of the Acoustical Society of America, **57**, 705-723.

- Winter IM, Robertson D and Yates GK (1990). Diversity of characteristic frequency rate-intensity functions in guinea pig auditory nerve fibres. Hearing Research, **45(3)** 191-202.

- Xie XM, Lancaster B, Peakman T and Garthwaite J (1995). Interaction of the antiepileptic drug lamotrigine with recombinant rat brain type IIA Na^+ channels and with native Na^+ channels in rat hippocampal neurones. Pflügers Archives, **430**, 437-446.

- Xie XM, Garthwaite J. (1996). State dependent inhibition of Na^+ currents by the neuroprotective agent 619C89 in rat hippocampal neurons and in a mammalian cell line expressing rat bain type IIA Na^+ channels. Neuroscience, **73 (4)** 951-962.

- Xie X and Hagan RM (1998). Cellular and molecular actions of lamotrigine: Possible mechanisms of efficacy in bipolar disorder. Neuropsychobiology **38**, 119-130.

- Yang N, George A and Horn R jr (1996). Molecular basis of charge movement in voltage-gated sodium channels. Neuron, **16**, 113-122.

- Yang N, George A and Horn R jr (1997). Probing the outer vestibule for a sodium channel voltage sensor. Biophysical Journal, **73**, 2260-2268.

- Young JS, Upchurch MB, Kaufman MJ and Fechter LD (1987). Carbon monoxide exposure potentates high-frequency auditory threshold shifts induced by noise. Hearing Research, **26**, 37-43.

- Yuen AW, Land G, Weatherley BC and Peck AW (1992). Sodium valproate acutely inhibits lamotrigine metabolism. British Journal of Clinical Pharmacology, **33(5)** 511-3.

- Zajiic, G. and Schacht, J. (1987). Comparison of isolated hair cells from five mammalian species. Hearing Research, **26**, 249-256.

- Zakrisson JE, Borg E, Liden G and Nilsson R (1980). Stapedius reflex in industrial impact noise: fatigability and role for temporary threshold shift (TTS). Scandinavian Audiology, **(Suppl 12)** 326-34.