Chapter 1

General introduction
Hearing is very important in everyday life. Humans depend on their hearing in a number of respects, such as for communication, socializing, learning, listening and to be warned for approaching danger. So when hearing is lost, it can have a disabling effect on a person’s life. It has been estimated that in the Netherlands > 1 million people have an average hearing loss of at least 35 dB at the frequencies involved in speech perception (1, 2, and 4 kHz). In general, two types of hearing loss can be distinguished: conductive and sensorineural hearing loss. Conductive hearing loss is due to a blockage of the anatomical cascade that conducts the sound waves from the outer to the inner ear. Examples are middle ear infections, perforation of the eardrum, and otosclerosis, a disorder in which the stapes may become immobile because of excessive growth of the bone. The other type of hearing loss is called “sensorineural” and refers to damage of the cochlea and/or auditory nerve. Sensorineural hearing loss can be induced by aging (presbycusis), loud music or noise, viral or bacterial infections and drugs (such as aminoglycoside antibiotics or the anti-cancer drug cisplatin).

In this thesis will be investigated whether the side effects of cisplatin upon the auditory system can be reduced or even prevented.

The peripheral auditory system

The peripheral auditory system can be subdivided into three parts: the outer, middle, and inner ear. The outer ear consists of the auricle and the external auditory canal and plays a role in sound localization, partly by frequency-selective modification of the sound wave, while it is transferred to the tympanic membrane (eardrum). Subsequently, these acoustic vibrations progress along the tympanic cavity (= middle ear) via the cascade of three tiny ossicles: the malleus (hammer), the incus (anvil) and the stapes (stirrup). Since there will be an energy loss when sound is transferred directly from air to the fluid in the cochlea, the ossicles amplify the sound and transfer the sound-induced vibrations via the stapes to the oval window, efficiently converting the sound waves into vibrations of the cochlear fluids.

The cochlea, which together with the vestibular apparatus comprises the inner ear (Fig. 1A) is responsible for the transduction of the sound-induced vibrations into electrochemical impulses in the auditory nerve. The cochlea consists of a fluid-filled spiraling tube that progressively diminishes in diameter towards the apex. In a cross section of the cochlea (Fig. 1B) the tube seems to consist of three scalae: the scala vestibuli, the scala tympani, and the scala media.
At its base, the scala vestibuli is sealed off by the oval window membrane, to which the stapes is connected. The scala tympani is closed at its base by another thin elastic membrane: the round window membrane. The scala tympani and the scala vestibuli are in open connection at the apex of the cochlea by an opening known as the helicotrema. In between these two compartments lies the scala media, which is separated from the scala vestibuli by Reissner’s membrane and from the scala tympani by the basilar membrane. On top of the latter is situated the organ of Corti, which contains the receptor cells: outer hair cells (OHCs) and inner hair cells (IHCs). TM: tectorial membrane.

Figure 1: A. The structure of the human inner ear, containing the vestibular apparatus and the cochlea. B. Cross section of the cochlea that shows the arrangement of the three scalae: the scala tympani (ST), scala vestibuli (SV) and scala media (SM). The stria vascularis (StV) is situated on the lateral wall of the scala media. SGN: Spiral ganglion cells; BM: basilar membrane; RM Reissner’s membrane. C. Detailed structure of the organ of Corti, which contains the receptor cells: outer hair cells (OHCs) and inner hair cells (IHCs). TM: tectorial membrane.

At its base, the scala vestibuli is sealed off by the oval window membrane, to which the stapes is connected. The scala tympani is closed at its base by another thin elastic membrane: the round window membrane. The scala tympani and the scala vestibuli are in open connection at the apex of the cochlea by an opening known as the helicotrema. In between these two compartments lies the scala media, which is separated from the scala vestibuli by Reissner’s membrane and from the scala tympani by the basilar membrane. On top of the latter is situated the organ of Corti, which contains ±16000 receptor cells (hair cells).

The scala tympani and scala vestibuli contain perilymph, which is like normal extracellular fluid in composition and is at or near ground potential. The scala media contains endolymph, which is more like intracellular fluid with high levels of K⁺ and low levels of Na⁺. K⁺ provides the major charge carrier for sensory transduction in the hair cells (Wangemann, 2002). The electrolyte composition and potential of the endolymph (± 80 mV) is regulated by an energy-consuming mechanism involving multiple ion transport processes in
the stria vascularis located in the lateral wall of the cochlea. The stria vascularis is a complex, multilayered structure, containing three layers of different cell types. Facing the endolymphatic space is a luminal layer of marginal cells, which are characterized by the presence of numerous basolateral membranes that are rich in mitochondria. The middle layer of the stria vascularis is composed of the capillaries and intermediate cells. Facing the spiral ligament there is a layer of multiple flat, basal cells.

The sensory epithelium of the inner ear, the organ of Corti, (Fig. 1C) is positioned on top of the basilar membrane. It contains two types of hair cells: the outer hair cells (OHCs) and the inner hair cells (IHCs). The OHCs are arranged in three rows and the IHCs in a single row. Both types contain a bundle of hair-like structures, the so-called stereocilia, on the surface facing the scala media. The tops of the stereocilia of the OHCs are inserted into holes in the tectorial membrane. When a sound-induced vibration reaches the cochlea, the basilar membrane moves up and down because of differences in the fluid pressure between the scala vestibuli and the scala tympani. This movement of the basilar membrane is accompanied by a shearing motion between the organ of Corti and the tectorial membrane, causing the stereocilia to bend. In response to these movements the hair cells generate a stream of electrical signals that code the frequency, intensity and duration of the sound. The electrical signals are generated in both IHCs and OHCs, but the neural information predominantly originates from the IHCs, which receive 90-95% of the afferent nerve fibers (Spoendlin, 1972). The signals are transported through the eighth cranial nerve (vestibulocochlear nerve) to the brain. The OHCs are responsible for the sensitivity and frequency selectivity of the cochlea. It is thought that OHCs can generate forces, by actively contracting and relaxing, enhancing the basilar membrane motion (Brownell et al., 1985).

Several factors, such as noise, bacteria, viruses, aging, drugs and other chemical agents, may cause hearing loss (ototoxicity). Some of the clinically applied ototoxic agents, such as aminoglycosides and cisplatin, have such a critical role in the treatment of serious, life-threatening diseases that the ototoxic risk can considered to be of less importance.

Cisplatin

The biological activity of cis-diamminedichloroplatinum (II) or cisplatin (Fig. 2) was discovered in 1965 by Rosenberg and co-workers during their studies to the effects of an electric current on bacterial growth. They noticed that an electrical field caused inhibition of Escherichia coli cell division (Rosenberg et
Further investigation indicated that the active agents responsible for this effect were platinum salts, which were produced at the electrode during electrolysis (Rosenberg et al., 1967). Several platinum complexes were tested for their biological activity and some of them, including cisplatin, suppressed cell division and induced filamentous growth of bacteria (Howle and Gale, 1970), which was known to be an indicator of DNA damage. Therefore, it was plausible to assume that cisplatin would also interfere with cell division in eukaryotes and subsequent studies revealed that cisplatin treatment indeed results in arrested growth of tumors. Surprisingly, the trans-iso-mer of cisplatin had no effect on tumor growth (Rosenberg et al., 1969).

The first clinical trials with cisplatin started in the 1970s. Nowadays, cisplatin is a widely used antineoplastic agent. Cisplatin-based combination chemotherapy displays significant efficacy in the treatment of testis tumors, ovarian carcinoma, squamous cell carcinoma of the head and neck, and non-small-cell carcinoma of the lung. This anti-tumor effect is due to a covalent binding between the platinum atom and genomic or mitochondrial DNA. Once cisplatin enters the cell the chlorine atoms are replaced by water, resulting in the formation of a positively charged aquated species that can react easily with nitrogen or sulphur atoms in intracellular macromolecules to form protein-, RNA-, or DNA-adducts. If there is another potentially reactive site nearby, cisplatin can react further to form intra- and inter-strand crosslinks (Kartalou and Essigmann, 2001), eventually leading to apoptotic (programmed) cell death of tumor cells. The clinical use of cisplatin, however, is limited by dose-dependent side effects, such as renal dysfunction, peripheral neuropathies, hearing loss, nausea, vomiting, and myelosuppression. Severe nephrotoxicity was the most important dose limiting finding in early clinical trials. With forced diuresis, this side effect has become more manageable, leaving peripheral neuropathies and ototoxicity as the major side effects of concern.
Cisplatin-induced sensory peripheral neuropathy

Peripheral neuropathy is one of the most commonly encountered side effects of cisplatin. It is dose dependent and may occur upon exposure to amounts as low as 150 mg/m² (Kopelman et al., 1988; Laurell and Borg, 1988). The extent of the neurotoxic effects is closely related to the total cumulative drug dose and dosage schedule, but it also depends on the concentration of the single dose administered. Significant neurotoxicity will always occur when patients receive more than 300 mg/m² cisplatin (Walsh et al., 1982; Cersosimo, 1989). The first clinical signs indicating cisplatin-induced peripheral neuropathy are numbness, tingling, loss of ankle jerks and painful paresthesia in the hands and feet. With further treatment loss of vibration sense, reduction in sensibility to touch or pain and decrease in position sense of the affected areas may develop (Thompson et al., 1984; Cersosimo, 1989). No damage to the motor system has been observed. The neurotoxic effect of cisplatin is limited to the sensory system (Roelofs et al., 1984; Thompson et al., 1984). Cisplatin-induced sensory neuropathy shows a typical delayed time-course, which often reaches its maximum 1-4 months after the last cycle of cisplatin chemotherapy (Hovestadt et al., 1992). Neurophysiological studies have demonstrated that cisplatin causes decreased amplitudes of the sensory nerve action potential, slowing down of sensory nerve conduction velocity and prolongation of sensory nerve latency (Cersosimo, 1989). Histological studies have shown sensory root ganglia disruption, loss of large myelinated fibers, axonal degeneration and degeneration of myelin sheaths (Roelofs et al., 1984; Thompson et al., 1984; Gregg et al., 1992). In animal studies, the electrophysiological and pathophysiological pattern of cisplatin-induced peripheral neuropathy is similar to that seen in patients. Cisplatin largely affects sensory nerve structure and function. Preferential toxicity is found for large-diameter neurons and proprioceptive sensory modalities, while motor nerves are spared (Muller et al., 1990; Apfel et al., 1992; Cavaletti et al., 1994; Cece et al., 1995).

Cisplatin-induced ototoxicity

Clinical studies

The ototoxic effect caused by cisplatin in humans is characterized by a bilateral, high-frequency sensorineural hearing loss (changes in thresholds at 4 to 8 kHz), usually associated with tinnitus. After prolonged drug use, hearing loss can progress to the speech frequency range, which is from 1-4 kHz (De Oliviera, 1989; Schweitzer, 1993). The incidence of cisplatin-induced ototoxicity ranges from 11 to 91%, depending on the mode of drug administra-
tion, dosage per treatment and cumulative dose (De Oliviera, 1989; Waters et al., 1991). Also, age and pre-existing hearing loss can influence the severity of cisplatin ototoxicity (Fausti et al., 1984). Bolus injections of 60 mg/m², administered once a week, have been shown to cause significant threshold differences after 6-12 months of treatment (Aguilar-Markulis et al., 1981). At cumulative doses of 270 mg/m² the first significant changes in auditory threshold appear, especially at the high frequencies (Schaefer et al., 1985). At doses of more than 450 mg/m², 88% of the patients show a high-frequency hearing loss (> 4 kHz) (McHaney et al., 1983). Only sporadically (incomplete) recovery of cisplatin-induced hearing loss has been reported (Aguilar-Markulis et al., 1981; Vermorken et al., 1983; Melamed et al., 1985; Laurell and Jungnelius, 1990). Histopathological studies in humans have shown loss of OHCs and IHCs in the basal turn of the cochlea, degeneration of the stria vascularis, significant decrease in the number of spiral ganglion cells, and damage to the cuticular plate (Wright and Schaefer, 1982; Strauss et al., 1983; Hinojosa et al., 1995; Hoistad et al., 1998).

**Experimental studies**

*In vitro* cisplatin-models generally concern the toxicity of cisplatin with respect to isolated cochlear OHCs (Saito et al., 1991, 1996; Sha et al., 2001; Devarajan et al., 2002) and cochlear explants (Clerici et al., 1996; Zheng and Gao, 1996; Kopke et al., 1997; Liu et al., 1998). However, most of the studies about the ototoxic effects of cisplatin have been performed *in vivo* in rodents: e.g., hamsters (Melamed et al., 2000; Kaltenbach et al., 2002), chinchillas (Ford et al., 1997; Tsukasaki et al., 2000), gerbils (Sie et al., 1997, 1999; Alam et al., 2000), rats (Laurell et al., 1995, 1997; Meech et al., 1998; Hatzopoulos et al., 1999, 2001, 2002), guinea pigs (Tange, 1984; Schweitzer et al., 1986; Kohn et al., 1988; Laurell and Engström, 1989, Laurell and Bagger-Sjöbäck 1991b; Schweitzer, 1993; Saito et al., 1994a, b; 1997a, b; Kohn et al., 1997; De Groot et al., 1997; Cardinaal et al., 2000a-c; Klis et al., 2000, 2002), and sporadically in other mammals such as dogs (Sockalingam et al., 2002) and rhesus monkeys (Stadnicki et al., 1975). In these studies cisplatin was administered by intraperitoneal injection at doses ranging from 0.75 to 4 mg/kg given repeatedly one to five times per week for a total of 1-8 weeks or as a single dose of 5-18 mg/kg by intraperitoneal injection or intravenous infusion.

The estimation of the onset of ototoxicity has been performed by measuring the auditory brain stem response (ABR), electrocochleography (ECoG) or by measuring the otoacoustic emissions (OAE). In animals the electrophysiological and pathophysiological pattern of cisplatin-induced ototoxicity is simi-
lar to that seen in patients. Cisplatin induces a dose-related permanent sen-
sorineural hearing loss starting at the high frequencies. Pathophysiological
studies in guinea pigs have shown that chronic cisplatin administration leads
to loss of OHCs, and at high doses also to loss of IHCs, with those in the basal
turn more severely affected than the ones in the middle and apical turns
(Nakai et al., 1982; Tange, 1984, Hoeve et al., 1988; Saito and Aran, 1994b;
Cardinaal et al., 2000a). Laurell and Bagger-Sjöbäck (1991a) have shown that
the morphological changes in the cochlea of guinea pigs after cisplatin expo-
sure occur in three stages. The first stage includes disturbance of the support-
ing cells surrounding the OHCs. The second stage was characterized by
degeneration of the OHCs; one of the first signs is loss of stereocilia and intra-
cellular vacuolation. The IHCs usually remain intact until all the OHCs have
degenerated. In the final stage collapse of the entire organ of Corti occurs. The
effects of cisplatin are not limited to the hair cells. Boheim and Bichler (1985)
have shown that cisplatin destroys the efferent auditory nerve fibers near the
OHCs. Others have found histological changes in the spiral ganglion cells of
guinea pigs, consisting of vacuolation of their cytoplasm (Cardinaal et al.,
2000b) and cell shrinkage (Van Ruijven et al., personal communication).
Furthermore, damage to the stria vascularis was observed in several studies in
rats and guinea pigs (Kohn et al., 1988, 1997; Meech et al., 1998; Campbell et
al., 1999; Cardinaal et al., 2000a, b). This damage consisted of blebbing and
vacuolation of the marginal cells and atrophy of the intermediate cells.
Besides these morphological changes, a smaller than normal endocochlear
potential (EP) was observed after administration of cisplatin to chinchillas
(Ford et al., 1997) and guinea pigs (Komune et al., 1981; Konishi et al., 1983;
A number of animal studies (Stadnicki et al., 1975; Nakai et al., 1982; Stengs et
al., 1997; Cardinaal et al., 2000b; Klis et al., 2000, 2002) demonstrated that sev-
eral of the cisplatin-induced ototoxic effects (OHC damage, increase of hear-
Summarizing, cisplatin seems to have at least three targets in the cochlea, the
organ of Corti, the stria vascularis and the spiral ganglion cells. Presently, the
relation between the respective effects on these targets, e.g., whether one is
causally related to the other and how these targets are involved in recovery, is
unknown.
Protection against cisplatin-induced side effects

Several attempts have been made to prevent cisplatin-induced side effects, *e.g.* by changing the dose, the method of administration or even by replacing cisplatin with a non-toxic analogue (*e.g.* carboplatin). However, these efforts proved to be unsatisfactory. Thus, another approach was investigated to overcome the toxic effects of cisplatin: pharmacological intervention. Several classes of compounds, such as neurotrophins and sulphur-containing compounds, have been found to protect against cisplatin-induced neuro- and oto-toxicity. In this section the most significant results from experiments with different classes of compounds that protect against cisplatin-induced ototoxicity will be reviewed.

**Fosfomycin**

The first agent tested for its possible protection against cisplatin ototoxicity was fosfomycin. Both Ohtani et al. (1985) and Schweitzer et al. (1986, 1993) showed that significantly less OHC loss occurs when animals are treated with 1 mg/kg/day cisplatin in combination with 300 mg/kg/day fosfomycin. However, these results could not be reproduced in later studies performed by Church et al. (1995) and Kaltenbach et al. (1997), in which higher doses of cisplatin (3 mg/kg/day, once every other day) were used in combination with fosfomycin co-treatment. These seemingly contradictory results could be explained by the observation of Ohtani et al. (1985) that at high concentrations of cisplatin (5 mg/kg/day) fosfomycin has no protective effect.

**Sulphur-containing compounds**

The application of sulphur-containing compounds was based on the hypothesis that damage caused by cisplatin is due to formation of free radicals, which interfere with the antioxidant defense system, resulting in oxidative stress (Rybak et al., 1995, Ravi et al., 1995). Severe oxidative stress produces major disruption of cell metabolism, resulting in cell death (Evans and Halliwell, 1999). Several compounds that may reduce free radical formation, have been described, *e.g.* sodium thiosulfate (STS), diethyldithiocarbamate (DDTC), 4-methylthiobenzoic acid (MTBA), L- and D-methionine, lipoic acid, and salicylate. In 1985, it was shown that sodium thiosulfate, already known to prevent cisplatin nephrotoxicity, prevents from neurotoxicity when administered simultaneously with cisplatin in patients (Markman et al., 1985). This compound also seemed to protect guinea pigs from cisplatin-induced hearing loss (Otto et al., 1988; Church et al., 1995; Kaltenbach et al., 1997; Saito et al., 1997a). However, it has been shown that STS reacts with cisplatin to form
covalently bound complexes, thus hampering cisplatin’s anti-tumor activity (Howell et al., 1982). Therefore, recent experiments have focused on the direct application of STS into the inner ear in order to selectively bind cisplatin in the cochlea (Wang et al., 2002). Another protective agent is the sulphur-containing amino acid methionine. Both the naturally occurring L-methionine and its synthetic analog D-methionine have been tested for their otoprotective properties. Campbell et al. (1996, 1999) have showed excellent protection by D-methionine from cisplatin ototoxicity in rats. Unfortunately, also L- and D-methionine lowered the systemic exposure to cisplatin (Reser et al., 1999; Ekborn et al., 2002; Vrana and Brabec, 2002). Therefore, topical administration of L- or D-methionine directly onto the round window membrane has been studied (Li et al., 2001; Korver et al., 2002). With this approach both compounds completely protect the inner ear from cisplatin-induced ototoxicity. Other sulphur-containing compounds that provide protection against cisplatin ototoxicity after systemic application are diethylthiocarbamate (Church et al., 1995; Kaltenbach et al., 1997; Rybak et al., 1995; Walker et al., 1994), 4-methylthiobenzoic acid (Rybak et al., 1997; Kaminura et al., 1999), salicylate (Li et al., 2002) and lipoic acid (Rybak et al., 1999a-c). However, excessive amounts of these compounds are necessary to realize a protective effect against cisplatin ototoxicity; the concentration of the sulphur-containing compounds exceeds the cisplatin dose by 5 to 100 times (e.g., 300 mg/kg D-methionine versus 8 mg/kg cisplatin). At these concentrations the sulphur-containing compounds are known to react directly with cisplatin resulting in a lowered cytotoxic effect of the drug (Ekborn et al., 2002).

**Neurotrophic factors**

In 1992 a group of neuropeptides was shown to have protective effects on cisplatin neuropathy (Apfel et al., 1992). These neurotrophic factors are, among others, nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF). Each of them signals through a specific high-affinity Trk receptor (Gao, 1999). NGF specifically acts on TrkA, BDNF and NT-4/5 on TrkB, and NT-3 selectively activates TrkC (Gao, 1999). All of the neurotrophins also bind to the NGF low-affinity receptor p75 (Chao, 1994). Three of these receptors, i.e., TrkB, TrkC, and p75 have been identified in spiral ganglion and hair cells (Ylikosky et al., 1993; Pirvola et al., 1994). Recent experiments have reported that rats and patients treated with cisplatin show a significant reduction in the level of circulating NGF (De Santis, 2000; Cavaletti et al., 2002). Since NGF suppresses the generation of reaction oxygen species (ROS)
(Dugan et al., 1997), reduction of NGF could result in an increased generation of ROS, eventually leading to peripheral neuropathy or ototoxicity. Most of the experiments with growth factors have been performed \textit{in vitro} with organotypic culture of cochlear explants or vestibular neuro-epithelia. BDNF and NT-4/5 were found to delay further degeneration of spiral ganglion cells. This protective effect, although somewhat smaller, was also observed when NT-3 was administered to spiral ganglion cells (Zheng et al., 1995, 1996). However, no attenuation of cisplatin-induced hair cell loss was observed with these compounds (Zheng and Gao, 1996). The results of studies performed with NGF are rather conflicting. Some authors have reported that NGF protects spiral ganglion cells from cisplatin-induced toxicity (Malgrange et al., 1994), while others consider NGF to be ineffective (Zheng et al., 1995, 1996). Kuang et al. (1999) recently showed that locally delivered GDNF protects guinea pig cochleas when it was administered in combination with systemically applied cisplatin. This is the only study known to show protection \textit{in vivo} by one of the above co-treatments at a dose in the µg-range, suggesting a specific mechanism against ototoxicity at the hair cell level. Recently, another group of peptides known as melanocortins has been demonstrated to protect against cisplatin-induced ototoxicity \textit{in vivo} at this dose range.

**Melanocortins**

Already in 1987, it was reported that the class of peptides known as melanocortins, is able to protect against cisplatin-induced neurotoxicity. Melanocortins are derived from the pituitary peptide AdrenoCorticoTropic hormone (ACTH) and include $\alpha$-Melanocyte Stimulating Hormone ($\alpha$-MSH), the ACTH$_{4-9}$ analog ORG 2766 and the synthetic cyclic peptide melanotan-II (MT-II) (Fig. 3). \textit{In vitro} experiments with neurons from the dorsal root ganglia (DRG) showed that both $\alpha$-MSH and ORG 2766 prevent the outgrowth-inhibiting action of cisplatin. However, they do not increase survival of DRG neurons nor do they appear to have any effect upon the death of supporting cells (Bär et al., 1993; Hol et al., 1994a; Windebank et al., 1994).

\textit{In vivo} experiments in rats showed a decrease in the sensory nerve conduction velocity (SNCV) as well as the number of thick myelinated fibers after treatment with cisplatin. Concurrent treatment with MT-II prevents the decrease of SNCV (Ter Laak et al., 2003), while ORG 2766 prevents both the decrease in SNCV (De Koning et al., 1987; Gerritsen van der Hoop et al., 1988; Hamers et al., 1991a, 1993a) and the decrease in number of thick fibers (Gerritsen van der Hoop et al., 1994).

A clinical trial, in which patients with ovarian carcinomas were concomitantly treated with cisplatin and ORG 2766, showed that ORG 2766 prevented part...
of the cisplatin-induced decrease of the vibration perception threshold (VPT) (Gerritsen van der Hoop et al., 1990). Unfortunately, 4 months after cessation of the cisplatin treatment the majority of patients still had abnormal VPT values and showed a continued increase of clinical complaints (numbness, loss of strength, pain, etc.). Nevertheless, these effects were less pronounced in patients previously treated with ORG 2766 (Hovestadt et al., 1992). In a more recent clinical study, in which more patients were included, this protective effect of ORG 2766 could not be reproduced; It was observed that ORG 2766 causes an increase in the onset and degree of neuropathies (Roberts et al., 1997).

Since the melanocortins α–MSH, ORG 2766, and MT-II showed promising neuroprotective effects in animal studies, we decided to test for the possible otoprotective action of these neuropeptides. Hamers et al. (1994) and Stengs et al. (1998b) showed a partially protective effect of 75 µg/kg/day ORG 2766 upon cisplatin-induced ototoxicity in guinea pigs as shown from changes in ECochG thresholds. Major threshold shifts were observed in all saline co-treated animals but not in all ORG 2766 co-treated animals after 8 daily doses of 2.0 mg cisplatin/kg (Hamers et al., 1994). Animals treated with ORG 2766 that showed
no protection, tended to have slightly worse CAP input-output curves than the saline co-treated ones, but OHC survival was significantly better in these ‘non-responders’ than in saline-treated controls (De Groot et al., 1997). Heijmen et al. (1999) demonstrated that treatment of albino guinea pigs with daily injections of cisplatin (2 mg/kg/day i.p. for 8 days) and concomitant injections of α-MSH (75 µg/kg/day s.c. for 9 days) results in a considerable number of animals with preserved hearing after cessation of cisplatin treatment. This was not found in the cisplatin/saline treated group. Thus, these experiments have demonstrated that ACTH-derived neuropeptides (melanocortins) are able to protect against cisplatin-induced ototoxicity. However, the mechanism by which these neuropeptides exert their otoprotective effect is not yet known. Their protective effect cannot be due to a direct interaction between the melanocortins and cisplatin, since both α-MSH and ORG 2766 were administered in much smaller doses than cisplatin. The actual target might be the intermediate cells in the stria vascularis. Meyer zum Gottesberge (2000) has suggested that these intermediate cells, which are actually melanocytes (Hilding and Ginzberg, 1977), are under α-MSH control. α-MSH may act as an emergency system in the regulation of inner ear homeostasis and function. ACTH-derived neuropeptides, such as α-MSH, are known to strongly bind to G-protein-coupled melanocortin (MC) receptors (Mountjoy et al., 1992). Therefore, α-MSH and ORG 2766 may exert their protective action by activating a MC-like receptor in the intermediate cells, thus preventing cisplatin from damaging the stria vascularis. Although the epidermal melanocytes have been shown to contain a MC-receptor that specifically binds to α-MSH (Tsatzmali et al., 2002), the presence of such a MC-receptor has yet to be demonstrated in the inner ear. Moreover, none of the known MC-receptors binds to ORG 2766. So the actual mechanism of action in the prevention of cisplatin-induced ototoxicity of the ACTH-derived neuropeptides is still unclear.

Methods for studying ototoxicity

In this thesis we are mostly interested in the prevention of cisplatin-induced ototoxicity. Cisplatin causes structural damage and functional loss in several tissues of the cochlea and the auditory nerve. In order to study these effects experimentally we have used two approaches: electrocochleography and histology.
Electrocochleography

Electrocochleography (ECochG) is a method to record the stimulus-related potentials of the cochlea and auditory nerve (Ruth et al., 1988). The response that is measured with ECochG occurs within the first 2-3 ms after an abrupt stimulus, and includes the following components (Fig. 4):

- The cochlear microphonics (CM), which is a stimulus-related alternating current (AC) potential that closely mimics the frequency of the stimulus. It is primarily generated by the OHCs, and it represents the displacement of the basilar membrane in response to an acoustic stimulus (Sellick et al., 1982). When the electrode is placed on the round window membrane the recordings reflect the activity of the OHCs in the basal turn.

- The summating potential (SP), a positive or negative stimulus-related direct current (DC) potential that reflects the time-related displacement of the cochlear partition. It is generated mostly by the IHCs, although a significant contribution is made by the OHCs (Durrant et al., 1998).

- The compound action potential (CAP), which is a transient response that is generated by peripheral eighth cranial nerve. It represents the summed response of the synchronous firing of thousands of auditory nerve fibers (Goldstein and Kiang, 1958; Ferraro et al., 1983). The amplitude of the CAP reflects the number of nerve fibers that are firing simultaneously.

Figure 4: Principles of an ECochG measurement. All recordings were performed at the round window. Traces obtained on stimuli with 180° phase difference are averaged separately. Adding these averages cancels CM so that CAP and SP are clearly distinguished. Subtracting these averages removes CAP and SP while the CM remains.
Histology

Another method to investigate cisplatin-induced cochlear damage is by studying the histopathological effects of the various functional entities of the cochlea, e.g. outer hair cells (OHCs), inner hair cells (IHCs), spiral ganglion cells and stria vascularis. In this thesis the quantitative analysis of OHCs and IHCs was performed in midmodiolar sections. The cochlea is divided into two halves after which 1 µm thick slices (sections) are taken from the cut surface (Fig. 5).

In these sections the number of hair cells were counted at seven different locations along the basilar membrane (2 transections for the basal turn; 2 transections for the middle turn and 3 transections for the apical turn).

The number of hair cells missing, relative to the expected 3 OHCs per transection, is a measure of cochlear damage. This hair-cell damage can be compared and correlated to the impairments found in the electrophysiological data.

Outline of the thesis

As outlined in the previous paragraphs, many advances have been made in the management of cisplatin-induced side effects. However, the neurotoxic and ototoxic side effects can not be treated effectively without hampering the cytotoxic action of cisplatin. Since ACTH-derived neuropeptides have shown to be effective as neuroprotective compounds that do not interfere with the antineoplastic properties of cisplatin, our group has tested these peptides for their...
possible otoprotective action. In previous studies, in which the drugs were administered during a fixed number of days, it was shown that both α−MSH and ORG 2766 have a beneficial effect on cisplatin-induced ototoxicity. However, since only part of the animals was protected, further optimization of the melanocortin treatment was considered to be necessary. Furthermore, the mechanism underlying the action of melanocortins had to be investigated.

This thesis is based on a longitudinal animal model in which an implanted electrode allows repeated measurements of cochlear sensitivity. In the first part the compounds are administrated systemically, in the second part we focus on a cochlear model in which the compounds can be delivered directly into the cochlea via a mini-osmotic pump system.

In the first study (Chapter 2) the longitudinal animal model is used to investigate the otoprotective effects of the new and more potent melanocortin-receptor agonist melanotan-II (MT-II). This synthetic melanocortin has been effective in the protection of cisplatin-induced peripheral neuropathy (Ter Laak et al., 2003). The objective of the study was to investigate whether MT-II is able to delay the occurrence of cisplatin-induced ototoxicity and to effect subsequent recovery. Animals were implanted with a permanent electrode and treated daily with cisplatin and MT-II until a 40 dB CAP threshold shift at 8 kHz occurred. Subsequently, cisplatin treatment was stopped and CAP recovery was studied for another 2 weeks.

A subsequent study was performed to test the protective effects of the melanocortin peptides α−MSH and ORG 2766 in the same longitudinal animal model (Chapter 3). We investigated whether these peptides delay the occurrence of the cisplatin-induced shift in auditory threshold, and whether they effect the subsequent recovery of the cochlear action potential (CAP) and endocochlear potential (EP). Both peptides showed significant ameliorating effects on the recovery of the CAP.

Since it was suggested that this recovery might be due to reversible strial failure and EP recovery, in the third study (Chapter 4) we have investigated the time course of EP recovery and whether the recovery of the EP is influenced by α−MSH co-treatment. In an experimental set-up similar to that of the second study, the EP and CAP were measured 1, 2, or 3 days after the criterion threshold shift at 8 kHz was reached.
To gain more insight into the mechanism underlying α-MSH action, the second objective of this thesis, we switched to the cochlear model in which effects of cisplatin and melanocortins could be studied without the possibly confounding influence of systemic administration. In the fourth study (Chapter 5) this animal model was used; cisplatin was administered directly into the cochlea via a mini-osmotic pump system while α-MSH or saline were administered daily by systemic injection. This approach decreased interanimal variability, which made it easier to quantify the efficacy of systemic α-MSH co-treatment. Furthermore, since cisplatin was delivered directly to the cochlea, any ameliorating effects of α-MSH would indicate that treatment with α-MSH probably involves a cochlear target.

To complement the previous study a mirror experiment was performed in the fifth study (Chapter 6). Guinea pigs that were implanted with a permanent electrode and a mini-osmotic pump, pumping either saline or α-MSH, were co-treated systemically with cisplatin until the 40 dB threshold shift at 8 kHz was reached. Then, cisplatin treatment was stopped, but intracochlear perfusion and electrocochleography were continued for 10 days to evaluate possible effects of local α-MSH treatment on recovery.