AUDITORY SENSE ORGAN DEVELOPMENT IN MICE AND THE ROLE OF TROPHIC FACTORS

BY

Marla J. Wilson

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Human Anatomy and Cell Science

University of Manitoba
Winnipeg, Manitoba

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

The neurosensory epithelium of the cochlear duct becomes the organ of Corti, a region specialized for sound detection. Neurotrophin-3 (NT-3) is essential for completing the development of the spiral ganglion and thus the afferent innervation of the sensory cells in the organ of Corti (Ernfors *et al.*, 1995). Colvin *(et al.*, 1996) demonstrated that *FGFR3* was pivotal for normal organ of Corti development. Using CD-1 mice, we have previously shown that localization of mRNA for fibroblast growth factor receptor 3 (*FGFR3*) was limited to a discreet region of the neonate murine organ of Corti. The aim of this study was to determine whether or not the presence of spiral ganglion cells and their afferent innervation to the sensory cells affects the expression of *FGFR3* in the organ of Corti. For these studies, mice deficient in NT-3 were used because they have reduced afferent innervation to the organ of Corti (Ernfors *et al.*, 1995). *In situ* hybridization, was used to compare *FGFR3* mRNA localization between NT-3 deficient mice and wildtype mice at ages E19 and P0. Localization of *FGFR3* appeared consistent in all genotypes. *FGFR3* mRNA was localized to the precursors of outer hair cells and supporting cells in the organ of Corti. These results suggest that the neurosensory epithelium and the tunnel of Corti start to form even with sparse or abnormal routes of innervation to the hair cells, and always in the presence of cells that synthesize *FGFR3* mRNA.

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Abbreviations

-/-	homozygous negative
-/+	heterozygous
+/+	wildtype
BDNF	brain derived neurotrophic factor
DEPC	diethylpyrocarbonate
dNTP	deoxynucleotide triphosphate
DTT	dithiothrietol
CN	cranial nerve
CNS	central nervous system
E*	embryonic day
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GER	greater epithelial ridge
IHC	inner hair cell
LER	lesser epithelial ridge
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NT	neurotrophin
OC	otic capsule
OHC	outer hair cell
P	pillar cell
P*	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SG	spiral ganglion
TC	tunnel of Corti
TE buffer	tris-EDTA buffer
TM	tectorial membrane

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1 Introduction

1.1 Hearing Disabilities and Deafness in Humans

1.1.1 Statistics

Approximately one out of every 2000 infants are born with some kind of hearing defect (Steel and Brown, 1994). As a special sense, hearing is ranked second only to sight in importance to man (Barr and Kiernan, 1988). In Canada, 5.5% of the population, or one million six hundred thousand people over the age of 15 years have a hearing disability. In Manitoba that percentage rises to 7.5% of the population (Statistics Canada, 1991).

1.1.2 Causes

Hearing damage can occur as a genetic pre-disposition, or as a result of injury. It is generally accepted that repeated insult to the organ of hearing, specifically the organ of Corti, with exposure to loud noises over an extended period of time will cause gradual hearing loss. Normal conversation, as measured in decibels, or units of sound pressure levels, creates a sound between 50-60dB. Long, repeated exposure to sounds of even 70dB (for example a hair dryer) can compromise hearing (National Institute on Deafness and other Communication Disorders, 1998). As life expectancy increases, and the population ages, we need to improve the quality of life of those who will become afflicted with hearing anomalies. Socially, hearing anomalies can cause depression and withdrawal, making it difficult for a person to function in a productive manner (Sobkowiez, 1997). The cost of treating hearing impairments is variable, and can be prohibitive. A hearing test

can cost thirty dollars, a hearing aid twenty two hundred dollars, and follow up appointments, for the treatment of the disability are difficult to assess (personal communication, Winnipeg Hearing Centre, 1999). Cochlear implant surgery is often considered, particularly for children, but is not always successful. Further, cochlear implant surgery is costly, about 25 000$ in the United States (American Academy of Otolaryngology, 1990). Extending longevity requires a responsibility to assure life can be continued in a manner that is acceptable and pleasant.

1.1.3 Classification

Hearing loss can occur via a variety of mechanisms: **conductive** when sound is not transmitted properly; **sensorineural** when sound is transmitted but cannot be processed; **mixed** a combination of transmission and processing problems; or **central** where the ear is functioning, but there is a problem in the central nervous system pathway (Shah and Halperin, 1982). Hearing loss can occur for a variety of reasons; for example hearing anomalies can occur as a symptom of a syndrome, such as Waardenburg syndrome (Jones, 1988), or as an anomaly unto itself. That is, deafness can be a symptom of a greater problem(s) or it can occur on its own. As such, classification of hearing loss is challenging. Genetically, there are at least sixteen conditions where deafness is the only symptom, and more than 150 conditions where deafness is one of several symptoms. Hearing loss can also be classified in terms of genetic or environmental factors. For example sensorineural hearing loss due to loud noises is environmental in origin, and sensorineural hearing loss due to Usher Syndrome is an autosomal recessive genetic

condition (Shah and Halperin, 1982). The majority of genetically linked deafness conditions are recessive in nature; however, autosomal dominant, and X-linked forms also occur (Shah and Halperin, 1982; Brown and Steel, 1994).

1.2 Development of the Mouse Inner Ear

1.2.1 An Overview of Adult Mouse Ear Gross Anatomy

An adult mouse ear consists of three regions: the outer (pinna, external auditory meatus, tympanic membrane); middle (ossicles: incus-malleus unit and stapes; and the auditory tube); and inner (vestibular and cochlear systems) (Kaufmann and Bard, 1999). The outer and middle ear function in the conduction and transmission of sound to the inner ear. The inner ear is found in the petrous portion of the temporal bone of the skull, and can be identified by a bulge called the tympanic bulla. The inner ear proper is comprised of a "bony labyrinth" filled with the sodium-rich perilymph fluid; and encases a "membranous labyrinth" filled with potassium-rich endolymph fluid (Salt, 1996). The two main components of the inner ear are; the vestibular system which is involved in balance; and the coiled cochlea which contains the spiral *organ of Corti* involved in hearing (Kaufmann and Bard, 1999; Walker and Homberger, 1992; Barr and Keirnan, 1988; Grant, 1978). The focus of this study is the *organ of Corti* in the cochlea (Figure 1).

1.2.2 Structure and Function of the Organ of Corti

The mouse cochlea is coiled upon itself one and one half to one and three quarter turns (in the human cochlea there are about two and a half coils) (Sher, 1971). The organ

of Corti contained in the cochlea is responsible for the transduction of sound to the spiral ganglion neurons. The spiral ganglion within the cochlea sends signals to the brain, which mediates the response we hear (Barr and Keirnan, 1988). The organ of Corti consists of supporting and sensory cells that work together to carry out the detection of sound waves. The organ of Corti rests on the basilar membrane; when sound is transmitted to the inner ear, this membrane vibrates, causing a cascade of effects. On top of the basilar membrane there is a layer of supporting cells, and on top of this a layer of hair cells. The hair cells are specialized sensory cells that, when stimulated, release neurotransmitters, (for example glutamate) (Eybalin, 1993), which "fire" the cochlear spiral neurons that send impulses to the brain. "Inner" and "outer" cells are named based on their position in relation to the modiolus, or medial part of the cochlea, and the tunnel of Corti. The hair cells have stereocilia protruding from the top of the cell. The tips of the stereocilia of the outer hair cells are embedded in the tectorial membrane (Hudspeth, 1997). The organ of Corti is centred around the tunnel of Corti; one row of inner and one outer row of microtubule-filled pillar cells function to support the tunnel of Corti. The organ of Corti contains two types of specialized sensory hair cells; inner and outer. In the mouse there is one row of inner hair cells along the length of the cochlea, and three rows of outer hair cells. It is thought that the inner hair cells are primarily responsible for sending the auditory information to the central nervous system, and the outer hair cells act as the cochlear amplifier, enhancing sounds (Hudspeth, 1997). When the cochlear amplifier starts to degenerate, implying outer hair cell injury, hearing loss commences (Hudspeth, 1997).

1.2.3 The Murine Auditory Pathway

The organ of Corti in the inner ear is innervated by **afferent** (carrying information TO the CNS from the ear) and **efferent** (carrying nerve impulses FROM the CNS to the ear) types of neurons. Primary sensory neurons (afferents) are found in the spiral ganglion of the cochlear part of the vestibulocochlear nerve (cranial nerve VIII). These bi-polar neurons have a "peripheral" process going to the hair cells of the cochlea, and a "central" process going to the CNS. The afferent central axons of the cochlea synapse at the cochlear nucleus and pass through three "synaptic relay stations" via the superior olivary nucleus, and the nucleus of the lateral lemniscus, before reaching the inferior colliculus. At the inferior colliculus, the auditory neurons synapse, and impulses are sent to the medial geniculate nucleus; from here, the impulses travel to the area of the brain that processes hearing, the temporal cortex, area one (Te1) as reviewed in Zilles (1985) (Webster, 1992).

Efferent or olivocochlear neurons arise from the olivocochlear nucleus and provide a "feedback inhibition", or a dampening function, to suppress afferent auditory input to allow the organ of hearing to respond more efficiently and acutely to sounds (Warr, 1992). A recognized efferent neurotransmitter substance is acetylcholine (Eybalin, 1993).

1.2.4 Innervation of the Cochlea and organ of Corti

Two types of **afferent** processes arise from the spiral ganglion of the vestibulocochlear nerve and innervate the hair cells of the organ of Corti: **type I** neurons

are myelinated and innervate the inner hair cells; **type II** neurons are not myelinated and innervate outer hair cells. These neurons of the spiral ganglion retain a unique embryonic condition of bipolarity. It is currently believed that approximately 95% of the neurons innervating the organ of Corti are type I, and 5% of the neurons are type II (Ryugo, 1992). This means extensive branching of type II neurons to innervate the more numerous outer hair cells. In an immature condition, multiple synapses per hair cell are formed, some of which will die back by the time the organ of Corti reaches maturity. In adults, both types of afferent neurons synapse directly with their respective hair cells. Adult inner hair cells have multiple afferent synapses per cell (type I), while outer hair cells have one afferent synapse per cell (type II). Adult Inner hair cells have several pre-synaptic efferent connections, but no direct efferent cell synapses. Conversely, mature efferent axons synapse directly with outer hair cells (Pujol *et al.*, 1998).

The coiled cochlea matures in a base to apex manner which also reflects the development of the organ of Corti and innervation patterns. The apex of the cochlea retains immature patterns of innervation, and of organ of Corti differentiation throughout the life of the animal (Pujol *et al.*, 1998).

1.2.5 Early Inner Ear Embryology

The ear is a structure that seems to have appeared several times over the course of evolution (Fritzsch *et al.* 1998; Manley and Köppl, 1998). There are different theories regarding the various aspects of ear development, and without further studies we will never be able to determine and understand the course of events that leads to an intricate,

fully functioning ear.

The mouse inner ear is derived from the surface ectoderm surrounding the developing hind brain (Fritzsch *et al.*, 1998). This thickening of the surface ectoderm is called the otic placode and in the mouse it appears at approximately day 8.5 post-conception (E8.5). It is thought that the otic placodes are induced to form and to invaginate by the hind brain, notochord and surrounding mesenchyme. Mesenchymally-derived fibroblast growth factor 3 (FGF3 or int-2) has been implicated as a factor involved in the induction of the otic vesicle (Fritzsch *et al.*, 1998). In addition, such molecules as retinoic acid and myosin VIIA, and such genes as pax2, hoxa1, dlx-3 (Fritzsch *et al.*, 1998), and Brn3.1 (Erkman *et al.*, 1996), just to name a few, are also important in the induction, spatial orientation, differentiation, and cell fate of the inner ear (Feteke, 1996; Morsli *et al.*, 1998). It is important to note that while we know these genes and molecules are important, we do not completely understand their functions, or the cascades and pathways with which they are associated. The otic placodes start to invaginate forming otic pits, and eventually bud off completely from the surface ectoderm forming otic vesicles. By embryonic day 9.5 there is a vesicle of neuro-ectodermal tissue surrounded by mesenchyme tissue from which the inner ear apparatus will develop.

1.2.6 Development of the Cochlea and the Organ of Corti

The cochlea starts to develop at E11 from the ventral part of the otocyst (Sher, 1971; Fritzsch *et al.*, 1998). By embryonic day 13, a cochlear duct can be identified (Sher, 1971). This duct continues to elongate and coil until embryonic day 18 (Sher,

1971). While this duct is forming, the epithelia of the cochlea and specifically the organ of Corti, start to arrange and differentiate in an organized manner. Between E15 and E16 the presumptive hair cells in the organ of Corti have stopped dividing. Location of the precursors of the cell types is difficult to establish early in development. The region of the developing organ of Corti that will give rise to the inner hair cells and its supporting cells is called the greater epithelial ridge (GER), while the region of the developing organ of Corti that will give rise to the outer hair cells and its supporting cells is called the lesser epithelial ridge (LER). At about E15, the location of a blood vessel in the organ of Corti; the vas spirale, and the shape, amount of cytoplasm, and location of the nuclei of the cells of the differentiating organ of Corti provide valuable clues as to which cell will differentiate into a hair cell, pillar cell, or supporting cell (Sher, 1971). Above the vas spirale is where the tunnel of Corti will form starting about E19 (Sher, 1971). This tunnel is flanked by one pillar cell on each side; from there, it is possible to extrapolate the locations of the precursors of inner and outer hair cells and supporting cells. According to Sher (1971) the developing hair cells have more cytoplasm than the supporting cells, while the cytoplasm of the supporting cells is more eosinophilic and their nuclei are more basally located. Based on morphological criteria, distinct inner hair cells develop slightly sooner than outer hair cells, at about E17 in the mouse (Pujol *et al.*, 1998). The basal turn of the cochlea differentiates first; therefore, it is the most mature part of the organ of Corti. In addition, the apex of the organ of Corti retains an immature form that persists in the adult condition.

The triggers that define what cell becomes what type are unknown. The leading

theory regarding the differentiation of hair cells is that first a cell becomes committed to developing into a hair cell, and then sends out inhibitory factors to prevent the cells surrounding it from becoming hair cells (Feteke, 1996; Morsli *et al.*, 1998). If this is in fact true, then two assumptions can be made; 1) all cell types in the organ of Corti have a common progenitor, and 2) there must come a point whereby the differentiation of the supporting cell prevents it from ever becoming a hair cell, for example in the event of hair cell injury. In the mouse, the organ of Corti does not become functional until postnatal day 10 (P10) or 11; that is, onset of hearing does not occur until this time. The fully tuned, functional murine organ of Corti develops by P21 (Corey and Breakefield, 1994).

1.2.7 Development of Cochlear Innervation

The neurons of the cochlear spiral ganglion, like the inner ear are derived from the otic placode (Fritzsch *et al.*, 1998; Pujol *et al.*, 1998). In the most mature part of the cochlea (basal turn), the neurons of the spiral ganglion cease to proliferate between E12 and E13 in the mouse. The differentiation of the spiral ganglion neurons starts at about E14 in the mouse. By E15 afferent nerve fibres are seen invading the murine cochlea. Efferent nerve endings start to invade the cochlea as early as E12 (Sher, 1971; Pujol *et al.*, 1998). Initially many nerve fibres can be detected invading the cochlea; however only a few of these will remain as the organ of Corti matures. As these nerve fibres reach their targets, the sensory hair cells of the organ of Corti, the phenomenon of nerve fibre die-back occurs. It seems that more nerve fibres than are ultimately required are generated and sent to the target (Pujol *et al.*, 1998). The target appears to provide trophic factors

for a certain number of fibres to persist, mature and form synapses with the target. Neurotrophin-3 (NT-3) is one neurotrophic factor shown to be important in the development of cochlear spiral ganglion neurons (Ernfors *et al.*, 1995). Synaptogenesis of the afferent and efferent nerves with the sensory cells of the organ of Corti commences postnatally (Pujol *et al.*, 1998). That is, the neurons do not fire or send and receive signals until after birth in the mouse.

1.3 Neurotrophin - 3

Neurotrophin 3 (NT-3) is a trophic factor of neurons; that is, it promotes growth, survival, development and maintenance of neurons (Fallon and Loughlin, 1993). It is a member of a family of trophic factors called neurotrophins. Other members of this family are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4) and neurotrophin 6 (NT-6) (Staecker *et al.*, 1996). NT-3 shares about 55% amino acid sequence homology with the other neurotrophins (Lindsay, 1994). NT-3 will bind with high affinity to the tyrosine kinase receptor trkC. NT-3 will also bind with low affinity to trkA and trkB which are high affinity receptors for the other neurotrophins. In addition, all of the neurotrophins will bind with low affinity to the receptor $p75^{NTR}$ (Lewin and Barde, 1996; Carter and Lewin, 1997).

Previous work using animals genetically engineered to have non-functioning NT-3, BDNF, NT-4 and NGF genes (Ernfors *et al.*, 1995, Fritzsch *et al.*, 1997a) suggests that NT-3 is an important trophic factor for the developing nerves of the cochlea, even more so than BDNF, and that NT-4 and NGF are not pivotal in cochlear development. In addition,

studies in rats (Wheeler *et al.*, 1994) have shown that NT-3 tends to have a higher expression in immature neurosensory structures, suggesting that it is an important trophic factor in cochlear development in embryonic stages. NT-3 is produced by the hair cells of the cochlea starting at E17 in the rat, which translates to about E15 in the mouse (Ylikoski *et al.*, 1993). NT-3 travels in a retrograde fashion to the neurons of the cochlear spiral ganglion (Wheeler *et al.*, 1994; Heymach and Barres, 1997), where the trkC receptor is found (Ylikoski *et al.*, 1993). The time of expression of NT-3 coincides with the start of afferent innervation from the cochlear spiral ganglion to the hair cells in organ of Corti (Fritzsch *et al.*, 1997).

1.3.1 NT-3 Knockout Mouse

The NT-3 "knockout" mouse generated by Ernfors (1993), carries a targeted disruption of the gene that codes for NT-3. In mice homozygous for this mutation (-/-) the NT-3 gene is not functional, and no NT-3 is produced. A large reduction in the number of neurons in the cochlear spiral ganglion has been observed by Ernfors (*et al.*, 1995) and later by Fritzsch (*et al.*, 1997). NT-3 (-/-) mice have severe cardiac defects that make postnatal survival impossible, except in a few rare cases (Donovan *et al.*, 1996). It is of interest to study the cochlear development in this animal model, because it provides the condition of reduced afferent innervation to the cochlea (Ernfors *et al.*, 1995; Fritzsch *et al.*, 1997). Since NT-3 appears to act as a target derived neurotrophic factor for neurites of spiral neurons, it reasonable to suspect that its absence will alter not only innervation pathways, but also other molecular and morphological features in the cochlea.

1.4 Fibroblast Growth Factor Receptor - 3

Fibroblast growth factor receptor 3 (FGFR3) is a member of the fibroblast growth factor receptor family (FGFR1 to 4)(Seddon *et al.*, 1995). This is a group of transmembrane receptors with an intracellular tyrosine kinase region and an extracellular region with three immunoglobulin like domains (Ornitz and Leder, 1995). The ligands for these receptors are fibroblast growth factors (FGFs), of which at least nine, and as many as 19 are known (Perez-Castro *et al.*, 1995; Seddon *et al.*, 1995). The effect of the ligand is dependent on the receptor with which it binds. The ligand-receptor interaction elicits a wide variety of cellular responses: for example, cell division and proliferation, trophic factor production and release, and a variety of developmental events (Colvin *et al.*, 1996). FGFR3 binds the ligand by simple dimerization of the receptors, or by dimerization of the receptors with a heparan sulphate proteoglycan mediator (Ornitz and Leder, 1992; Pickles *et al.*, 1998). At this point the receptor prompts tyrosine kinase activation. It is not known what cascades or pathways this activation initiates. Further, the mechanism behind the cellular responses is not understood.

Other studies have shown that FGFR3 is imperative for normal development of the mouse inner ear (Colvin *et al.*, 1996). In FGFR3 "knockout" mice, no proper pillar cells form, and no tunnel of Corti forms. As a result, adult FGFR3 knockout mice are profoundly deaf.

The FGFR3 gene in the mouse is approximately 15kb in length, and contains 18 introns and 19 exons. The FGFR3 protein contains three extracellular Immunoglobulin - like domain loops, coded by exons one to nine. Exons ten and eleven code the

transmembrane and juxtamembrane portions of the receptor, exons eleven to eighteen code the intracellular kinase region; and exons eighteen and nineteen code for the C-terminal. FGFR3 is characterised by two variants produced by alternative splicing, FGFR3 IIIb and FGFR3 IIIc. The IIIb isoform binds only aFGF, while the IIIc isoform binds aFGF and FGF4 with high affinity, binds bFGF and FGF5 with low affinity (Ornitz and Leder, 1992; Perez-Castro *et al.*, 1995) and potentially binds with FGF8 and 9 (Pickles *et al.*, 1998).

1.5 Objectives

Damage to sensory systems is characterized by injury to the sensory input structures, and then by a regression of the nerves that innervate them (Staecker *et al.*, 1996). The inner ear is no exception; if the hair cells of the organ of Corti in the cochlea are compromised, then their nerves fibres die back. This presents a two-fold problem; 1) repair of the sensory structures (the cochlear hair cells), and 2) re-innervation of these structures. As such, it is reasonable to assume that an animal model with absent or abnormal innervation to its sensory structures may also exhibit anomalies in the neurosensory cells of theses structures. The mammalian auditory epithelium has not been shown to repair itself, in contrast to findings in avian and amphibian models (Staecker and Van De Water, 1998). To date, little is known regarding the molecular mechanisms involved in normal hair cell development, knowledge of which would provide valuable clues to the problems of repair and regeneration in the auditory system. By using a mouse model with abnormal inner ear innervation, we will gain insight into the importance of

innervation in regards to organ of Corti development, and will determine if lack of innervation results in an absence, up-regulation, down regulation, or redistribution of FGFR3, known to be pivotal for inner ear development (Colvin *et al.*, 1996). FGFR3 mRNA was first localized to the developing murine organ of Corti using *in situ* hybridization by Peters (*et al.*, 1993). However cell types expressing this receptor were not identified.

NT-3 knockout mice do not produce NT-3 (Ernfors *et al.*, 1995). In an undisrupted developing rat inner ear, NT-3 is produced by the developing outer and inner hair cells of the organ of Corti from second week embryos through adult ages (Pirvola *et al.*, 1992, Ylikoski *et al.*, 1993, Wheeler *et al.*, 1994). Previous work has shown that NT-3 deficient mice have a substantially reduced afferent innervation to the organ of Corti (Ernfors *et al.*, 1995). This reduction of neurons in the spiral ganglion, would presumably result in a reduced production of aFGF, the ligand for FGFR3 in the organ of Corti (Pirvola *et al.*, 1995). First we seek to localize the cells that synthesize FGFR3, and are thus responsive to aFGF. Then we can ask: does the absence or reduction of ligand, or other factors from the processes of the spiral neurons, alter the cells that would normally produce FGFR3?

It is important to determine which cells in the organ of Corti are synthesizing FGFR3 mRNA. If FGFR3 is expressed preferentially on cochlear hair cells as opposed to the cochlear supporting cells, a difference in cellular development and function could be identified and targeted for further study. Thus, localization of FGFR3 mRNA is an essential step in understanding its role in the developing inner ear. Since FGFR3,

conferring responsivity to aFGF, appears to be a differentiated feature of some of the cells of the neurosensory epithelium, could its absence be used as a molecular indicator of incomplete development in mice with cochlear abnormalities?

A greater understanding of the development of the auditory system will provide clues that can be applied to finding a mechanism for sensory epithelial repair, regeneration and protection. The answers in the developmental patterns of the auditory system could impact other areas of neuroscience and potentially have exciting new clinical applications - for example the protection of damaged nerves, or the regeneration of regressed nerves.

Questions for this Study

1) In which cells of the organ of Corti is the FGFR3 mRNA localized?

2) Does the absence or delay of innervation to the organ of Corti disrupt the distribution patterns of FGFR3 mRNA?

2 Methods and Materials[1,2]

2.1 Animal Housing and Breeding

For this study 6 *NT-3* heterozygous mice (2 males, 4 females) were obtained from Jackson Laboratories (Bar Harbour, Maine, USA) and a small breeding colony was maintained in the Dentistry housing facility of Central Animal Care Services at the University of Manitoba. These mice were derived from Ernfor's (1995) original colony. These *NT-3* mice had a targeted disruption of the *NT-3* gene, and as such had three possible genotypes: wild-type (+/+, both copies of the *NT-3* gene are active), heterozygous (-/+, one copy of the gene is active and the other is inactive), or mutant (-/-, both copies of the gene are inactive). Breeding of heterozygous mice yielded the mutants we wanted to study. Mutant, or NT-3 deficient mice survive gestation, but generally die within 24 hours of birth. In addition we were able to obtain wildtype mice to be used as controls, and heterozygous mice, which were used both for control tissue and to maintain the colony.

Mouse pregnancies were timed by placing two heterozygous (-/+) females together with one heterozygous (-/+) male overnight. Should a plug be seen in the female the following morning, it was counted as day 0.5 of gestation. The gestation period of these mice is approximately 21 days. Potential mothers were monitored to ensure pregnancy was progressing, as the presence of a plug does not always guarantee a pregnancy. Timed matings are essential, so that sick (NT-3 deficient) mice can be identified as soon as

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[1]For a complete solutions guide see appendix I
[2]For a complete reagents guide see appendix II

possible after birth and euthanised. In addition timed matings are essential for embryonic studies so we know the age of the embryos taken.

Mice that were not euthanised at birth were used to expand the NT-3 colony. At the time of weaning, mice were ear - tagged with an identification number so that an inventory could be monitored, tail and blood samples taken for genotyping, and gender and colour noted (appendix III).

All protocols were done in accordance with, and approved by the local animal care protocol committee.

2.2 Tissue Collection

2.2.1 Pre-Euthanasia Survey

Adults

Before euthanasia adult +/+ and -/+ mice (except for timed pregnant females) were evaluated for abnormal limb movements and markings. In addition, the presence or absence of the mouse's Preyer's reflex was noted. Two keys were hit together to create a loud metallic sound. If the mouse moved the pinna of its ear, a positive Preyer's reflex was recorded, no movement of the pinna, a negative Preyer's reflex.

Age P0

Neonatal mice were also examined for abnormal limb movement. If the neonate had spastic limb movement, and had difficulty moving in general, it was a possible mutant. Further, if the neonate had no obvious milk in its gut (at this age the skin is transparent

and the presence of milk in the gut can be observed)(Figure 2) and small stature, it was a possible mutant.

2.2.2 Anaesthetic

Neonatal and adult mice were deeply anaesthetized with the veterinary gas AErrane (1-chloro-2,2,2-trifluroethyl difluromethyl ether, [isoflurane])(OHMEDA #402-224-00, DIN 02091267). Liquid AErrane was poured onto a cotton ball in a jar with a screw top lid. A metallic drain stopper was placed over the cotton so the animal would not come into direct contact with the anaesthetic. The animal to be euthanised was placed in the jar for about 2-3 minutes, or until completely anaesthetized. A toe-pinch test was done to make sure animals were properly anaesthetized, and a nose cone with AErrane gas was near by in case animals started to re-gain consciousness at any time during the procedure.

2.2.3 Weight and Crown-Rump Length

All animals were weighed on a Mettler BasBal (BB2440) digital scale (except for timed pregnant females and their embryos); crown-rump length of unfixed embryos and neonates was measured using a stainless steel ruler and recorded in millimetres.

2.2.4 Collection of Tissues for Genotype Analysis

Tail Collection

Tails of embryos, neonates, and weanlings were collected by using clean scissors

that were wiped with 0.2N HCl (Anachemia #R-2830D, UN-1789) and rinsed with autoclaved reverse osmosis water.

Embryonic and neonatal tails were collected in full, and frozen in sterile eppendorf tubes.

Weanling tails were taken at the time of ear tagging, at approximately three weeks of age. Approximately 1.0 cm of the weanling's tails were cut and frozen in sterile eppendorf tubes.

Blood Collection

At neonatal and weanling stages blood samples were collected in the following manner: 1) *Neonatal*; at the time of perfusion, when the chest cavity was opened, blood samples were taken on clean Guthrie card strips, placed in sterile 15mL centrifuges tubes left with the top off overnight to allow the blood to dry. Clean scissors (wiped with 0.2N HCl and washed with autoclaved reverse osmosis water to prevent DNA cross-contamination) were used for each animal and gloves were changed between animals. 2) *Weanling* blood samples were taken at the time of ear tagging and tail collection. When the tail was sampled with a clean pair of scissors and forceps, the blood that was produced was spotted onto a clean Guthrie card strip and left to dry in the same manner as above. The cards were then processed in the same way. Blood samples were not taken from mice at fetal stages because usually an insufficient amount of blood was present to test.

Embryonic Liver Collection

At embryonic ages, liver was also collected for genotype analysis. Blood was not easy to collect, and in order to have a second tissue to be used as a backup, liver was collected. The abdominal cavity was opened, and using clean forceps and scissors (as outlined above) a portion of liver was dissected out and frozen in sterile eppendorf tubes.

2.2.5 Tissue Perfusion and Fixation

Neonatal mice were perfused by an intracardiac route using gravity pressure with approximately 15 mL phosphate buffered saline (PBS, pH 7.2), followed by approximately 35 mL 4% paraformaldehyde (diluted in PBS) for about 10 minutes. The mice were decapitated, skin removed from the skull, and the skull opened to expose the brain to fixative, and immersed in 4% paraformaldehyde at 4°C overnight.

Embryonic mice were obtained by removing the mother's uterus at the time desired (for example embryonic day 15.5, or 18.5 as per section I) and excising the embryos from the uterus and amniotic sacs. This procedure was done in cold PBS. Embryonic mice were not perfused, but they were promptly decapitated, skull opened up the mid-line to expose the brain, and immersed in cold paraformaldehyde. They were kept at 4°C in 4% paraformaldehyde overnight.

2.2.6 Tissue Dissection and Preparation

Brains were removed from the skull, the tympanic bulla and base plate of the skull (basisphenoid bone) identified, and the excess tissue removed to leave only the middle and

inner ear portions. The brain was bisected and saved for paraffin processing. The ears were separated, the left one was used for paraffin processing, and the right one for frozen processing. A few ear samples were embedded in JB-4 solution (Polysciences Inc., Warrington, PA) and used for plastic sections.

Routine paraffin processing was done using the Technotron. Before freezing, cryo-protection of tissue for frozen processing was done by immersing the tissue in sequential solutions of 10%, 20%, and 30% sucrose (Mallinckrodt #AR8360) in 4% paraformaldehyde until the tissue sank. Then the tissue was rinsed for several hours in 30% sucrose in PBS. The tissue was embedded in OCT Compound (Tissue-Tek #4583) using 10mmX10mmX5mm biopsy Cryomolds (Tissue-Tek #4565) and frozen at -50°C in isopentane (2-Methylbutane, ACROS Organics #12647-0010) that was pre-cooled on dry ice. Frozen tissue blocks were stored at -70°C until cut.

2.2.7 Tissue Sectioning

Frozen

RNase free conditions were used at all times. Wearing unpowdered latex gloves, frozen sections were cut at a 10μM thickness using a *Shandon* Motorised Cryotome with a *Leica* C-cryostat knife. Cryostat chamber temperature was set between -19°C and -25°C depending on the ambient humidity; the higher the humidity, the lower the temperature. Four to six sections were placed on Polylysine (Esco #P-4981) or Superfrost Plus (Fisher #12-550-15) microscope slides. Approximately every sixth section was saved on a reference slide for routine hematoxylin and eosin staining. This reference slide was used

to landmark which area of the ear was being used. Slides to be used for *in situ* hybridization were dried on a slide warmer set at 42°C (Fisher) and stored in slide boxes with DRI-RITE™ at -70°C.

Paraffin

Paraffin sections were cut at a thickness of 5μM, and floated onto microscope slides using a water bath set at 41°C containing autoclaved double distilled deionized water and dried on a slide warmer. RNase free conditions were maintained. Sections were cut using disposable microtome blades (Fisher #12-631R, No. R35). Slides to be used for *in situ* hybridization were stored in slide boxes at room temperature.

Plastic

Plastic sections were cut at a thickness of 3μM with glass knives. Sections were floated onto plain glass slides using a room temperature water bath. They were then dried on a slide warmer and stained with hematoxylin and eosin. Plastic sections were not used for *in situ* hybridization, so RNase free conditions were not employed.

2.3 Genotype Determination

In order to accurately determine the genotype of the NT-3 mice, several methods of sample DNA preparation were attempted. All preparations were analysed using polymerase chain reaction (PCR) technology.

2.3.1 Sample Preparations

Blood Spot Preparation

Blood spot analysis was done with the help of Dr. Yvonne Myal according to her published procedures (Gregory *et al.*, 1995). The blood dried on Guthrie cards was prepared for PCR analysis in a consistent manner.

Two eppendorf tubes (500µL) per animal were prepared. One blood spot was excised from the Guthrie cards with a paper punch ($1/8^{th}$ inch hole size) into each tube (2 per animal). Between animals the hole puncher was sterilized by soaking it for 2 minutes in 0.2N HCl, rinsing with double distilled water, drying the hole puncher with a Kimwipe, and punching out several clean holes of Whattman filter paper. Autoclaved toothpicks were used to manoeuvre the blood spots in certain cases. Gloves were changed between samples and a clean circle of filter paper was put under the punching area in case the punch fell out.

The samples were then fixed by pipetting 100µL of methanol (Fisher Scientific #A452-1, UN 1230) into each eppendorf tube, and the tubes were checked to ensure that the blood spot was covered with methanol. The blood spots were methanol fixed for 15 minutes, the methanol removed via pipette (using clean tips for each sample), and dried in a vacuum dessicator overnight. Tubes were closed and stored at room temperature until PCR analysis was done.

Genomic DNA Preparation and Analysis

Genomic DNA preparation was carried out using a phenol/chloroform extraction

protocol provided by Dr. Yvonne Myal. Mouse tails were placed in sterile eppendorf tubes, cut up, and 200μL of "mouse tail solution" (appendix I) was added; a final concentration of 1.75mg/mL proteinase K enzyme was added to the tubes. Care was taken to ensure that clean instruments were used for each sample. Tubes were closed and samples were incubated overnight at 55°C in a shaking water bath. The next day phenol was added to the tubes (as much as the tube could hold), and tubes were inverted approximately 100 times. Tubes were spun in a microfuge for 5 minutes. The aqueous phase and the interphase were removed and transferred to a new tube. To this new tube as much 1:1 phenol:chloroform as the tube could hold was added. The tubes were inverted and spun in a microfuge as previously described. Then the aqueous phase was transferred to a new tube. This time only chloroform was added, and the tubes were inverted and spun down as previously described. The chloroform step was repeated. An equal volume of isopropyl alcohol was added to the tube, which was then gently inverted until a stringy white precipitate formed - this precipitate was the genomic DNA. The precipitate was transferred to an eppendorf tube containing 1.0mL 70% ethanol, and was gently shaken at 4°C for 1-2hours. The 70% ethanol was poured off, and 1.0mL 100% ethanol was added, the tube was shaken gently and spun down for 2 minutes. The 100% ethanol was poured off and the DNA pellet was dried for about 5 minutes using the Speedvac. Once the pellet was dry, 50μL TE buffer (pH 8.0) was added to the tubes, and the tube was "flicked" until the DNA came away from the side, it was left overnight at 4°C.

The DNA concentration was measured using the optical density (OD) value at 260nm wavelength on the spectrophotometer (Milton Roy Spectronic 1001 plus). This

value was used to calculate the DNA concentration in ng/μL. The DNA was diluted so that when 1μL was added to 49μL of PCR solution, the final amount of DNA was 50-100μg total. Genomic DNA was always stored at 4°C.

Homogenate Preparation

The homogenate method of sample preparation was modified after a protocol from Hogan *et al.* (1994). Using sterile techniques, 40μL of 10XPCR homogenate solution (appendix I) was added to each tail sample. 500μg/mL proteinase K was added to each sample, and they were incubated in a 55°C water bath overnight. 1.0μL of this solution can be used for PCR, or a 1:10 dilution can be made.

Each method works fine, however I selected to use the former procedure as I found there was less cross contamination and it was faster to use 1.0μL of straight homogenate. Homogenates were frozen at -20°C until they were analysed.

2.3.2 Polymerase Chain Reaction (PCR) Analysis

Three PCR primers provided by Dr. Shiu were made using the sequence provided by the Jackson Laboratory web site (http://lena.jax.org/re...protocols/Ntf3_KO.html). Primers *oIMR130* and *oIMR131* were used to amplify the wild-type allele identified by a band at the **250bp** position, and primers *oIMR130* and *oIMR132* were used to amplify the mutant allele identified by a band at the **350bp** position on an ethidium bromide-stained agarose gel (appendix IV).

50μL PCR reactions were set up using PCR buffer, 10mM each of dNTP-4's (A,

G, T, C[Pharmacia #27-2035-01]), 50μM primer oIMR130, 50μM primer oIMR131 *OR* primer oIMR132 (depending on which allele [wild-type or mutant] was amplified), 50ng of DNA, *or* 1μL of homogenate, *or* one fixed blood spot from the animal that was genotyped, and 2.5 units total of *Taq* polymerase (Pharmacia #27-0799-01). Pharmacia *Taq* polymerase reaction buffer contains $MgCl_2$, which yielded a 1.5mM final concentration in a 50μL reaction. Two PCR reactions per animal were carried out, one reaction for each allele that could be present (appendix V). PCR amplification was done using the profile:

7 minutes at 94°C

30-35 cycles for DNA or homogenate, 40-45 cycles for blood spots
1 minute at 94°C denaturing
1 minute at 55°C annealing
1 minute 30 seconds at 72°C extension

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15 minutes at 72°C
Park at 4°C

(Thermocycler: GTC-2 Genetic Thermal Cycle, Precision Scientific)

2.3.3 Visualization of PCR Products by Agarose Gel Electrophoresis

A horizontal gel electrophoresis apparatus was used (Gibco-BRL Horizon 58). A gel containing 25mL of 2% agarose in 1xTBE buffer, with approximately 0.5μL of ethidium bromide (10mg/mL) was set at room temperature. Two tubes of PCR product were generated for each animal, one to identify the normal allele, the other the mutant allele. 3μL of each PCR product from the same animal were placed in one tube with 1μL of general loading buffer (total sample volume = 7μL). A 100bp molecular marker ladder

(Pharmacia #27-4001-01) was run along side the samples (1μL [1μg/μL] ladder in 5μL 1xPCR buffer and 1μL general loading buffer) so that the size of the PCR product could be evaluated. These samples were run on the gel at 60 volts for 1.5 - 2 hours. Visualisation of DNA bands was done on an ultraviolet light box, and a picture taken using a digital camera (CCD72 series camera, Dage-MTI inc.) and the MCID program (Microcomputer Imagining Device, M4, Imaging Research Inc.).

A **single** band at the **250bp** position indicates a **wild-type** mouse (normal complement of *NT-3* alleles), **one** band at the **350bp** position indicates a **mutant** mouse (no active *NT-3* alleles present), and **two** bands (one at the 250bp and one at the 350bp position indicates a **heterozygous** mouse (one active and one in active copy of the *NT-3* allele). Results were printed out on a *Hewlett Packard laser jet IIIp* printer (Resolution Enhancement PCL5) (Figure 3).

2.4 Localization of *FGFR-3* Using *in situ* Hybridization

FGFR-3 messenger RNA (mRNA) was localized to cells using an ^{35}S-labelled riboprobe designed to detect *FGFR-3* mRNA in the mouse. The anti-sense probe was an ^{35}S-labelled RNA strand that was the complement to the *FGFR-3* mRNA in the mouse cells; thus they would bind together, forming a stable hybrid. A variety of controls were used. A sense control match was done with every anti-sense experimental slide. That is, for every slide used to detect FGFR-3, a serial slide was used to apply a probe with the same sequence as the FGFR-3, thus it should not bind to the mRNA in the tissue. A "no probe" control was done which involved applying non-radioactive hybridization buffer (i.e.

buffer without probe) to serial sections of anti-sense and sense slides. This served as a negative control, where, with no probe applied, no radioactive signal would result. For each probe an RNase control run was done. This control involved using the RNase A enzyme to destroy RNA in certain tissue sections. Two sets of slides were analysed, one set that contained intact RNA and one that had the RNA destroyed. Where the RNA was destroyed, no radioactive signal was produced on either the anti-sense or sense slides, but in the normal experimental condition (with intact RNA) a radioactive signal was produced only on the anti-sense treated sections. The results obtained from the control slides indicated that the probe was indeed binding to RNA, and not DNA or protein (i.e. no RNA, no anti-sense signal). RNase free conditions were maintained throughout the procedure. That is, clean, DEPC (diethyl pyrocarbonate, Sigma #D5758) treated solutions, baked glassware, and autoclaved instruments were used wherever possible.

2.4.1 Preparation of Riboprobe

Template and Cloning

The *FGFR-3* riboprobe was constructed using the PCR primer sequences published by Ornitz and Leder (1992). The probe was designed for the transmembrane-juxtamembrane portion of the *FGFR-3* RNA, spanning exons 10 and 11 which is the conserved area. Thus, all isoforms of the *FGFR-3* RNA would be detected using this probe. Total RNA was extracted from mouse brain, and reverse transcription polymerase chain reaction (RT-PCR) was used to amplify the sequence specific to *FGFR-3*. Using a Promega Kit, this 429bp cDNA sequence was cloned in a *pCR II* (Invitrogen Corporation)

plasmid vector.

Probe construction

The anti-sense probe was constructed by linearizing the cloned plasmid with *XbaI* restriction enzyme (Boehringer Manneheim #67257), and transcribing the DNA to RNA using *SP6* RNA polymerase (Boehringer Manneheim #810274), incorporating ^{35}S-labelled UTP (ICN Pharmaceuticals Inc., 1000Ci/mmol) as one of the bases.

The sense probe was constructed by linearizing the cloned plasmid with *Bam HI* restriction enzyme (Boehringer Manneheim #220612) and transcribing it using *T7* RNA polymerase (Boehringer Manneheim #881767) and ^{35}S-labelled UTP as was done for the anti-sense probe.

The anti-sense and sense probes were 549bp and 527bp in length respectively. The β-emissions from the radiolaelled probe were counted using 1μL of a 1:10 dilution on a liquid scintillation counter (Beckman LS6500 multi-purpose scintillation counter). These counts were used to calculate the appropriate dilutions of the probe to be used in hybridization buffer to generate the radioactive hybridization solution (appendix II).

Probe Dilution

Probe dilution follows the procedure of Simmons (*et al.*, 1989). The amount of probe needed to produce a desired concentration is estimated through calculations (appendix I). Desired probe concentration used for these calculations was $1.0x10^6$CPM/mL. Generally, probe concentrations of $1.0x10^6$CPM/mL were used for

paraffin sections, and $5.0x10^5$CPM/mL for frozen sections.

The equation used for probe desired was a theoretical estimate; a count of the radioactivity in the hybridization solution was done to determine the actual probe concentration. Before use, a 5.0 µL sample of hybridization solution was counted using a scintillation counter and the probe concentration was adjusted as required to remain as consistent as possible.

2.4.2 *In situ* Hybridization Procedure (Angerer and Angerer, 1992; Simmons *et al.*, 1989)

Pre-Hybridization Treatment

All Pre-hybridization and Hybridization steps were done using RNase free conditions, and steps were performed at room temperature unless otherwise noted. Frozen sections were warmed to room temperature before the storage box was opened. Paraffin sections were deparaffinized using two changes of xylol (stirring) for ten minutes each. Sections were re-hydrated using a graded series of ethanols (100%, 95%, 70%, 50%, 30%) for three minutes each and a quick rinse in DEPC-treated water.

Thawed frozen and deparaffinized sections were pre-hybridized with a quick fix in 4% paraformaldehyde (one minute), rinsed in PBS (two changes at one minute each) and a 0.0001% proteinase K (Sigma #P0390) treatment of 30 minutes at 37°C, re-fixed for one minute in 4% paraformaldehyde, rinsed twice for one minute each in PBS, and acetylated with 0.25% acetic anhydride (Mallinkcrodt # un1715, FW 102.09) for five minutes with 0.1M TEA (triethanolamine, 2,2',2''-Nitrilotriethanol, Hydrochloride, $C_6H_{15}NO_3 \cdot HCl$, FW

185.7, Sigma # T-9534) in DEPC water to balance tissue charges. Slides were rinsed two times in 2xSSC, and finally dehydrated in a series of cool ethanols (70%, 95%, and three changes of 100%). Slides were dried for approximately one hour at room temperature before the hybridization step.

Hybridization

The probes were heated at 65°C in a water bath for 10 minutes. This unanneals the probe and makes it available for binding to the tissue sections. The probes were then quenched on wet ice, quickly spun down, and 8µL DDT(5M) (Dithiothreitol, Cleland's Gibco # 15508-013) added. Anti-sense *or* sense probe were applied to the appropriate sections as follows: 1) The size of coverslip needed to cover the sections was determined, 2) the amount of probe used was based on the size of the coverslip (eg. 30x22mm coverslip = 30 µL probe), 3) the probe was pipetted onto the coverslip in a crescent shape, 4) the slide with the tissue sections was slowly lowered onto the coverslip at an angle in order to avoid air bubbles. The slides with the coverslips and probes were placed into a humid chamber (a Tupperware container lined with paper towels soaked in sterile 2xSSC and a plastic separation between the paper towel and the slides that allowed the SSC to circulate). The humid chamber was sealed with Parafilm, carefully placed in a Ziplock freezer bag, and placed in a 55°C water bath overnight (14-20 hours).

Post-Hybridization Washes

The purpose of the post hybridization treatment of the slides was to reduce

background signal by "washing away" unbound probe. Slides were removed from the humid chamber, and coverslips were floated off in a solution of 2xSSC with 0.01M DTT. Slides were quickly rinsed in four consecutive solutions of 2xSSC with 0.01M DTT. Then slides were washed three times in 2xSSC + 0.01M DTT for ten minutes each, and the solutions were shaken gently on a motorized shaking platform (Red Rotor). Following this, slides were washed in a solution of 50% formamide in 2xSSC plus 0.01M DTT at 55°C (hybridization temperature) for 30 minutes. Slides were then rinsed two times in 2xSSC ***without*** DTT for five minutes each, gently shaking. (It was necessary to temporarily remove the DTT in preparation for the RNase A step because RNase A is inactivated by DDT). Slides were exposed to RNase A (Boehringer Mannheim #109169) at a concentration of 0.02mg enzyme per mL RNase buffer for 30 minutes at 37°C. (RNase A digests single stranded RNA, therefore it should reduce unbound probe and hence reduce background). Slides were washed at room temperature in three changes of 2xSSC + 0.01M DTT, gently shaking for ten minutes each, and one bath of 1xSSC + 0.01M DTT, gently shaking for five minutes. Slides were then treated in 0.1xSSC + 0.01M DTT at 55°C for 25 minutes, followed with a room temperature solution of 0.1xSSC + 0.01M DTT for 5 minutes. Dehydration of the sections was done by treating them in 50% ethanol in 0.1xSSC + 0.01M DTT and 70% ethanol in 0.1xSSC + 0.01M DTT, followed by 95%, and three changes of 100% ethanol for three minutes each. Slides were dried at room temperature in preparation for the autoradiography detection step.

2.4.3 Autoradiography (Paterson, 1983)

Detection of the *FGFR-3* riboprobe was carried out in the dark by dipping the slides in undiluted, melted Kodak NTB-2 (Interscience #1654433) liquid autoradiographic emulsion at 42°C, drying them for at least two hours in a humid chamber and sealing the slides in a light tight box, and allowing exposure for 7-21 days (depending on the radioactivity of the probe, and thickness of the tissue section) at 4°C. A set of slides with controls was developed using Kodak D-19 (#146 4593) developer at 16°C (diluted 1:1 with double distilled de-ionized water), and Kodak Fixer (#123 8146). Sections were either rinsed and counter stained immediately, or rinsed, dried, and then re-hydrated for counter staining. Sections were counter stained using modified Harris hematoxylin (no acetic acid, no mercury) (Fisher Scientific #SH30-500D).

2.4.4 Microscopic Observations and Photographic Analysis

Slides were analysed using a Nikon Microscope with a Nikon Camera attachment. Sections were observed in light and dark field conditions, and pictures were taken at different magnifications using both colour and black and white film.

3 Results

3.1 Animal Model

3.1.1 Observations

Embryonic Day 19

At embryonic ages, mice are more difficult to evaluate with respect to their phenotypical characteristics compared to neonate (P0) ages. Grossly, mutant mice tend to appear smaller than their wildtype and heterozygous littermates.

Histologically, all of the components that one would expect to find in a wildtype mouse organ of Corti are present in the NT-3 -/- mouse organ of Corti (Figure 4). That is, the Greater and Lesser epithelial Ridges of the developing organ of Corti are present, their cells appear to be developing and do not show any gross morphological anomalies.

Postnatal Day 0

Shortly after the time of birth, litters were inspected. Generally, neonates that presented a phenotype including a small size, no milk in the stomach, cyanotic colour, and spastic/awkward movements were mutant in genotype. Comparatively, wildtype and heterozygous mice appeared larger, had ingested milk (Figure 2), were pink in colour and showed more co-ordinated movements.

Histologically, all of the components present in the organ of Corti of wildtype mice are also present in the NT-3 -/- mouse organ of Corti at P0 (Figures 5 and 6). Developing inner and outer hair cells, developing inner and outer pillar cells, the

developing tunnel of Corti, developing supporting cells, and developing tectorial membrane, were compared in NT-3 wildtype and -/- mice organ of Corti at P0. However, it appeared that the NT-3 -/- had a reduced number of cochlear spiral ganglion neurons, as compared to a wildtype or heterozygous counterpart (Figure 7).

3.1.2 Birth Weights and Sizes

Birth Weights

Individuals that were born to heterozygous matings were selected for study initially based on their small appearance. Therefore the sampling was not random and the Mann-Whitney rank sum test analysis was used. Mice with the mutant genotype had a statistically significant lower birth weight as compared to their wildtype counterparts (Table 1). Males with the -/- genotype had a statistically significant lower birth weight than males with the +/+ genotype (Table 2). However no significant difference was noted between the birth weights of female mice compared in the same manner (Table 2). In addition, no significant difference was detected between the birth weights of males and females of the same genotype (Table 2).

crown-rump length

Using our method of measuring by ruler only, no significant difference was observed between the crown-rump lengths of mutant, wildtype and heterozygous mice (Table 3).

3.2 NT-3 Mouse Colony General Data

Starting with our six founder NT-3 mouse colony members, we generated 110 mice using heterozygous matings. From Table 4, it was observed that fewer than one quarter of the offspring were mutant. We generated an additional 27 mice using wildtype - heterozygous matings (Table 5), and 28 mice using wildtype matings for control tissue. Not all breedings resulted in pregnancy. In terms of breeding success of heterozygous mating pairs, a pregnancy rate of at most 68% was achieved in our study. All mice particulars (for example, date of birth, genotype, parents) can be traced via a database created for the NT-3 mouse colony (appendix III).

This strain of mouse appears to have a longevity of one to one and a half years, and mice heterozygous for the NT-3 gene do not appear to have their life span compromised, as we have successfully bred heterozygous individuals beyond one year in age (male 3951 at 13 months, and female 3978 at 14 months).

3.3 Blood Spots vs DNA Extraction vs Homogenate Analysis

Animals were genotyped using the polymerase chain reaction (PCR) technique (Figure 3). Three methods of DNA preparation were tested; blood spot analysis, phenol-chloroform genomic DNA extraction, and homogenate technique. The homogenate preparation approach proved to be the most efficient and accurate of the three.

3.4 *In situ* Hybridization for FGFR3 mRNA

Fifteen *in situ* hybridizations for FGFR3 mRNA were performed on NT-3 tissue of

various ages and genotypes. In total 176 slides were used: 32 (14 -/-, 12 +/+, 6 -/+) at age E19 and 144 (60 -/-, 46 +/+, 36 -/+) at age P0. To summarize, at age E19 nine different animals were tested, 3 -/-, 4 +/+, and 2 -/+ (Table 6a), and at age P0 fifteen different animals were tested, 7 -/-, 3 +/+, and 5 -/+ (Table 6b). Initially, paraffin and frozen sections were both used, but paraffin sections yielded better resolution, and were used wherever available. A standardized procedure and a probe radioactivity of about $1.0x10^6$CPM/mL was used in all experiments.

3.4.1 FGFR3 mRNA Expression in CD-1 and NT3 Mice

FGFR3 mRNA localization in CD-1 mice from ages E15.5 to P3 is summarized in Table 7. FGFR3 mRNA was localized to the pre-bone cartilage of the otic capsule, and to the developing neuroepithelial cells of the cochlear duct. No signal for FGFR3 mRNA was found in the vestibular system (Figure 8). A comparison between the localization and expression "level" of FGFR3 mRNA between the CD-1 strain and the NT-3 (Balb/c) mouse strains at ages E19 and P0 was conducted. No difference was detected in the localization of FGFR3 mRNA between the NT-3 wildtype and CD-1 mouse strains in the developing organ of Corti at ages E19 and P0 (Figures 4 and 9). FGFR3 mRNA as indicated by radioactive signal, was localized to the two tiers of neuroepithelial cells in the presumptive organ of Corti of the cochlear duct, and also to cells in the pre-bone cartilage of the otic capsule. The FGFR3 mRNA signal appeared to be predominately localized over the developing outer hair cells, developing outer pillar cells, and to a lesser extent over the supporting cells (Figures 4 and 9).

3.4.2 FGFR3 mRNA Expression in NT3 Wildtype, Heterozygous, and Mutant Mice

Embryonic Day 19

At embryonic day 19 (E19) no difference was observed in the localization of FGFR3 mRNA between NT-3 wildtype, heterozygous, and mutant mice. The signal presented consistently over the two tiers of neuroepithelial cells, in all three genotypes (+/+, -/+, and -/-) similar to that found in the CD-1 mouse strain at this age (Figure 4).

Postnatal Day 0

At postnatal day 0 (P0), no difference was observed in the localization of FGFR3 mRNA between NT-3 wildtype, heterozygous, and mutant mice. The signal presented consistently over the two tiers of neuroepithelial cells in all three genotypes (+/+, -/+, and -/-) similar to that found in the CD-1 mouse strain at this age (Figures 9 and 10).

3.4.3 FGFR3 mRNA Localization Differences Between E19 and P0 NT-3 Mice.

Mice of the same genotype were compared at ages E19 and P0. Overall no difference in the level of FGFR3 mRNA expression between NT-3 mice (-/-, -/+, and +/+ genotypes) was detected at ages E19 and P0. Possibly a lower intensity of signal is demonstrated over the supporting cells, or fewer supporting cells are labelled in the organ of Corti at age P0, as compared to the organ of Corti at age E19 (Figures 4, 9 and 10).

4 Discussion

Embryonic Day 19

By appearance alone, embryonic mice were difficult to evaluate based on their phenotypic characteristics. Size and weight measurements were not taken, because it was essential to immerse the embryos in fixative to preserve their inner ears, the focus of this study.

Postnatal Day 0

Neonatal mutant NT-3 mice are easier to identify than embryonic mutant NT-3 mice, but their identities still must be confirmed through genotyping. Statistically, a significant difference was found between the birth weights of mutant and wildtype NT-3 mice (Tables 1 and 2). Mutant NT-3 mice had a lower birth weight than wildtype mice. One reason for this is that NT-3 affects other functions and areas of the body in addition to cochlear spiral neurons, proprioceptive sensory, and sympathetic neurons. It has been shown by Donovan (*et al.*, 1996) that NT-3 deficient mice have serious heart defects, similar to those found in human Tetralogy of Fallot patients. Both the heart defects and locomotor feedback defects could account for the early death of the mutant neonatal mice. However, heterozygous NT-3 mice possess milder heart defects that go undetected and can allow the mouse to live a seemingly normal life.

For genotyping the tail homogenate technique was the most efficient and reliable method of DNA analysis. DNA extraction by the phenol-chloroform method was time

consuming and resulted in cross contamination from other samples. Since it was difficult to collect enough blood from the embryonic NT-3 mice, and the blood from the chest cavity was often too diluted, the blood spot technique was not a reliable option.

Inner Ear Histology: Embryonic Day 19 & Postnatal Day 0

As seen in figures 4, 5 and 6, all of the components of the developing organ of Corti appear to be in place in all genotypes of the NT-3 mice at ages E19 and P0. These data suggest that the absence of NT-3 did not directly affect the development of the gross morphological features of the organ of Corti. However, it appears that the NT-3 deficient mouse has a reduction in the number of cochlear spiral ganglion neurons (figure 7). This observation is compatible with the results of Ernfors (*et al.*, 1995) and Fritzsch (*et al.*, 1997) who found an 87% deficit in number of cochlear spiral ganglion neurons compared to the number in a wildtype animal. Fritzsch (*et al.*, 1997) has also suggested that the height of the neuroepithelial ridges in the developing organ of Corti differs between wildtype and NT-3 deficient mice. This height difference could reflect an incomplete differentiation of the organ of Corti in mice that are NT-3 deficient. To measure the height of neuroepithelial ridges requires plastic embedding and semi-thin sectioning, as well as consistent planes of section of cochlear ducts. Plastic embedment could not be used for post-section *in situ* hybridization in our study.

Sympathetic and some other sensory and motor neurons of NT-3 (-/-) mice are also compromised as they are dependent on NT-3 during development (Fariñas *et al.*, 1994; Ernfors *et al.*, 1994; Kucera *et al.*, 1995; ElShamy *et al.*, 1996; Liebl *et al.*, 1997;

Wyatt *et al.*, 1997).

FGFR3 mRNA Expression in CD-1 and NT-3 Mice

For this study six FGFR3 riboprobes labelled with ^{35}S were made and used under the same conditions. The "half-life" of ^{35}S is about two months, so it was important to keep the probes new, and the radioactive counts of the aliquots used similar, to allow for reliable comparison between groups. Thus, degradation of the probe did not become a factor confounding comparisons. In addition, care was taken to standardize the tissue being processed and to run different ages and genotypes in the same solutions and to dip them in the same batch of emulsion. When analysing the slides it was important to note the location of the cochlear coil, because the basal coil develops faster than the middle coil, which develops faster than the apical coil. As such, the basal cochlear coil was chosen for this study (Figure 1).

Since the gross anatomy, and the inner ear histology of the NT-3 mutant mouse appears largely normal, it was of interest to examine one of the biochemical properties of he inner ear, namely, expression of FGFR3 mRNA. FGFR3 is a receptor with a restricted distribution in the inner ear (Peters *et al.*, 1993). In the rat cochlea it has been shown that the mRNA for aFGF, a ligand for FGFR3, is first detectable in the cochlear spiral ganglion neurons in late fetal development. aFGF mRNA appears later in the stria vascularis, and inner and outer hair cells of the organ of Corti (Luo *et al.*, 1993). Further, work by Pirvola (*et al.*, 1995) has shown that the destination for neuronally derived (i.e. from cochlear spiral ganglion neurons) aFGF is the organ of Corti.

In the present work, FGFR3 was localized in the CD-1 and wild type NT-3 mouse to the developing outer hair cells and outer pillar cells specifically, and possibly to a lesser extent to the inner pillar cells and outer supporting cells.

Ernfors (*et al.*, 1995) demonstrated with the NT-3 knockout mouse that NT-3 was required for the survival of a full complement of cochlear spiral neurons. It was not conclusively shown if the surviving spiral neurons of the NT-3 deficient mice were of type I (innervating the inner hair cells of the organ of Corti) or type II (innervating the outer hair cells of the organ of Corti). Since Pirvola (*et al.*, 1995) showed the organ of Corti to be an important destination for aFGF from the spiral neurons (most of which require NT-3), we speculated that the aFGF "pathway" was interdependent with the NT-3 "pathway" during development. With the reduction in the number of neurons in the NT-3 deficient mouse, the trophic effect of neuronal processes on the neurosensory epithelium in the organ of Corti might also be significantly affected. This reduction could also result in a retarded differentiation of the organ of Corti.

Our results supported an independence of the aFGF and NT-3 trophic cascades. In our study FGFR3 mRNA was localized to the same cell types in the organ of Corti, regardless of the presence of NT-3 during development. As figure 10 shows, the expression of FGFR3 mRNA was not qualitatively different between inner ears of wild type and NT-3 deficient mice.

Fritzsch (*et al.*, 1997) has proposed that there is a compensatory process taking place in the cochlea of the NT-3 deficient mouse. That is, the few spiral neurons that remain are sufficient to innervate the ear by what we might consider abnormal or

unconventional routes. Ernfors (*et al.*, 1995) has shown that a BDNF/NT-3 double knockout mouse presents no cochlear spiral ganglion neurons, as opposed to the few spiral ganglion neurons that remain with a NT-3 knockout alone. This suggests that both trophic factors work in tandem to create the environment necessary for conventional neuron development and innervation. This is supported by the "Max Factor" work done by O'Conner and Tessier-Lavigne (1999). Their studies demonstrate that Maxillary growth factor, the substance that guides the maxillary branch of the trigeminal nerve to its target, is part NT-3, part BDNF.

In addition, it is possible that the few neurons that survive the harsh trophic-deficient conditions produce enough aFGF to support the cells of the organ of Corti, or that another ligand is acting through this FGFR3 and eliciting the same, or at least a similar intra-cellular response. Further, neurons have the potential to switch their affinity for specific neurotrophins over the course of development and maturation (Davies, 1997). That is, as the neuron develops, its trophic needs change. The phenomenon of "neurotrophin switching" makes the clues to neuron development all that more elusive. One would have to trace the expression of several neurotrophins, and attempt to pinpoint when the "switch," if any, occurs, and why that switch in neurotrophin affinity benefits the neuron population in question.

Validity of Techniques

Radioautography using a specific ^{35}S-labelled riboprobe provided quite precise information as to the location of FGFR3 mRNA. However, due to the electron kinetics

or "scatter effect" of this isotope, the grains that are formed in the emulsion overlying our tissue can not resolve an exact cellular location. Thinner sections would improve resolution, but cannot entirely compensate for grain scatter. Supporting cells in the organ of Corti have very narrow cellular processes that lie adjacent to the hair cells. Distinguishing which cell type (hair cell, supporting cell, or both) is labelled requires further studies; electron microscopy with a tritium labelled probe would help solve the resolution problem.

The identity of the cells, for example the pillar cells, is based on location alone and at times, particularly at younger ages, it is difficult to distinguish between certain cell types. It would be beneficial to employ a double labelling technique where one or more cell types could be positively identified. This method would allow the identification of the cells radiolabelled with FGFR3 to be more accurate.

The ideal condition for this study would have been a mouse model that had the spiral ganglion neurons obliterated. Unfortunately in the NT-3 knockout mouse a small number of the neurons remained, and as such could have greatly affected the cochlear development.

Future Studies

It would be of interest to study the other ligands that could be present in the inner ear and that have the potential to act through FGFR3. Recently Pickles (*et al.*, 1998) found that the only splice variant of FGFR3 in the inner ear is IIIc. The identification of a splice variant specific to the inner ear is of interest because it restricts the list of FGFs (in

addition to aFGF) that could bind to the FGFR3 in this region. For example FGF4 can act through FGFR3 IIIc and has a role in embryonic development (Ornitz and Leder, 1992; Perez-Castro *et al.*, 1995; Pickles *et al.*, 1998). Perhaps ample non-neuronal aFGF is present in the NT-3 knockout mouse. As mentioned in "validity of techniques," a double labelling technique that would allow for certain identification of one or more cell types in addition to the localization of the FGFR3 mRNA in the organ of Corti would provide valuable information pertaining to the role of FGFR3 in the murine cochlea.

Significance

While we by no means have all the steps in the developmental cascades of the inner ear, relationships can be deduced by studying cellular morphology and biochemistry in different transgenic mouse models. The present study provides information about inner ear development, with the long term goal of solving the regeneration dilemma, and eventually, of designing appropriate therapies that could be applied clinically in auditory protection, or regeneration.

Conclusions

From our results, it appears that the NT-3 deficient mice have fewer neurons and have abnormal routes of innervation, as also shown by Fritzsch (*et al.*, 1997). Despite this, there was no discernable effect on the localization of FGFR3 in the developing organ of Corti. In basal coils of the cochlea, the tunnel of Corti appeared to be opening all our newborn mice (Figure 10). This observation is compatible with the results of Colvin (*et*

al., 1996), indicating that FGFR3 is essential for the formation of the tunnel of Corti. Therefore we assume that there was functional FGFR3 present in the organ of Corti of NT-3 deficient mice. Our work suggests that the neurosensory epithelium and the tunnel of Corti start to form even with sparse or abnormal routes of innervation to the hair cells, and always in the presence of cells that synthesize FGFR3 mRNA. Our results are also compatible with the evidence from organ culture by Dazert *(et al.*, 1998) who has shown that FGF-receptor is present in more than one cell type in the developing organ of Corti. Finally our results suggest that during development cochlear cells which synthesize FGFR3 mRNA are not dependent on events that require NT-3.

Figures

Figure 1: Morphology and location of the developing cochlea.
Age Embryonic Day 17 CBA mouse, coronal orientation (see inset*), frozen section, 10μm thick, H & E stain, 35X magnification.

Note the location of the otic capsule (oc) and the cochlear coils (A, B, M). The modiolus will form where the cochlear nerve (CNv) is seen invading the Cochlea. The organ of Corti develops in the cochlear duct.

BSp Basisphenoid Bone, CNv Cochlear Nerve, OC Otic Capsule, A - Apical Cochlear Coil, B - Basal Cochlear Coil, M - Middle Cochlear Coil.

(*reproduced from MRC (UK) and the University of Edinburgh, http://genex.hgu.mrc.ac.uk/Databases/Anatomy/Diagrams/ts26/ts26_1.gif)

Superior

Medial

Lateral

Inferior

Figure 2: Neonatal NT-3 wildtype mouse with translucent skin displaying the milk in the stomach. Refer to the inset* for scale and body orientation.

(*reproduced from MRC (UK) and the University of Edinburgh, http://genex.hgu.mrc.ac.uk/Databases/Anatomy/Diagrams/ts27/ts27_1.gif)

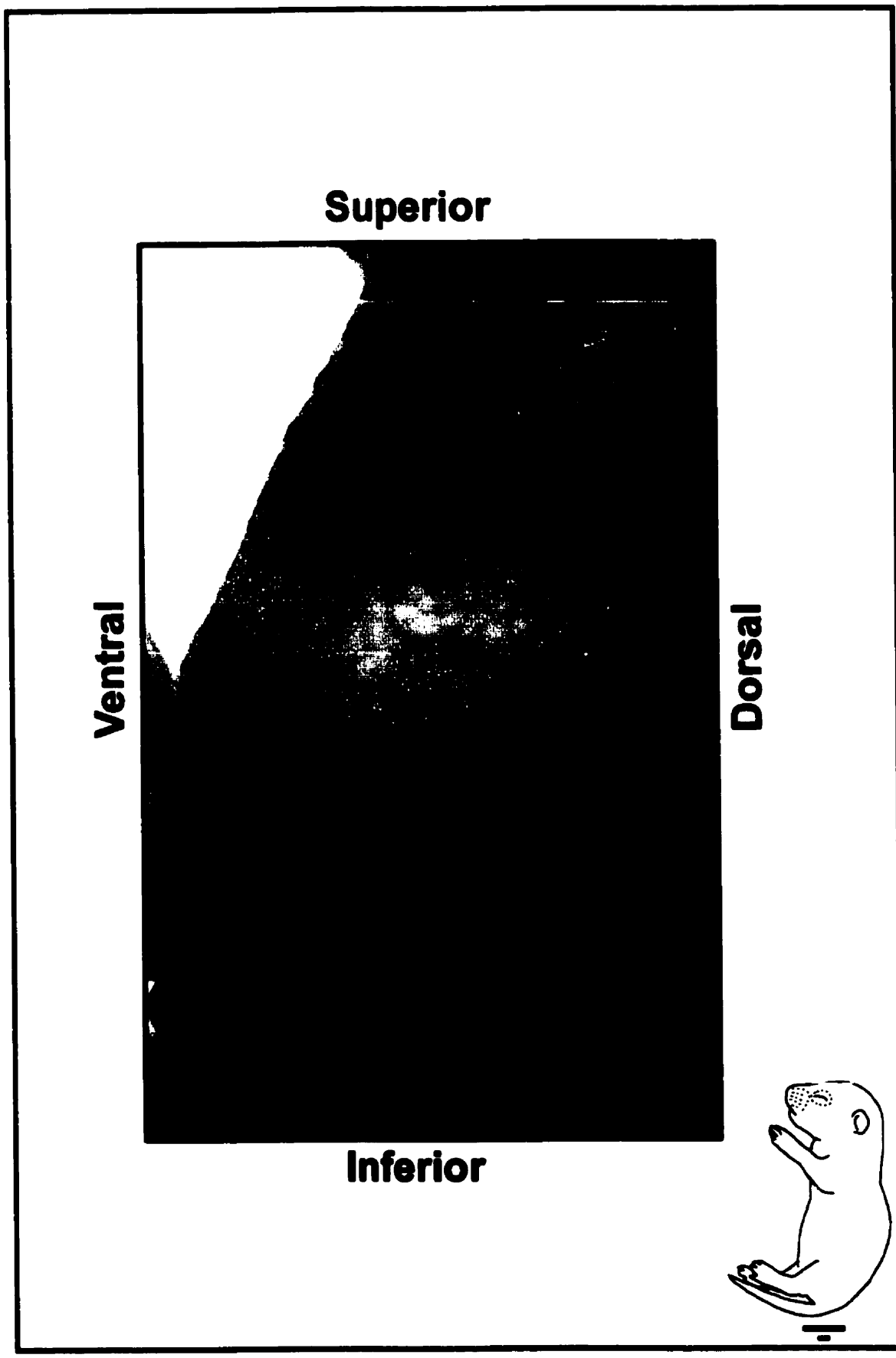

Superior
Ventral
Dorsal
Inferior

Figure 3: An example of NT-3 mouse genotype analysis of PCR products using agarose gel electrophoresis.

One band at the 250bp position indicates a wildtype mouse (two active copies of the NT-3 allele), one band at the 350bp position indicates a mutant mouse (no active copies of the NT-3 allele), and two bands (one at the 250bp and one at the 350bp positions) indicate a heterozygous mouse (one active and one inactive copy of the NT-3 allele).

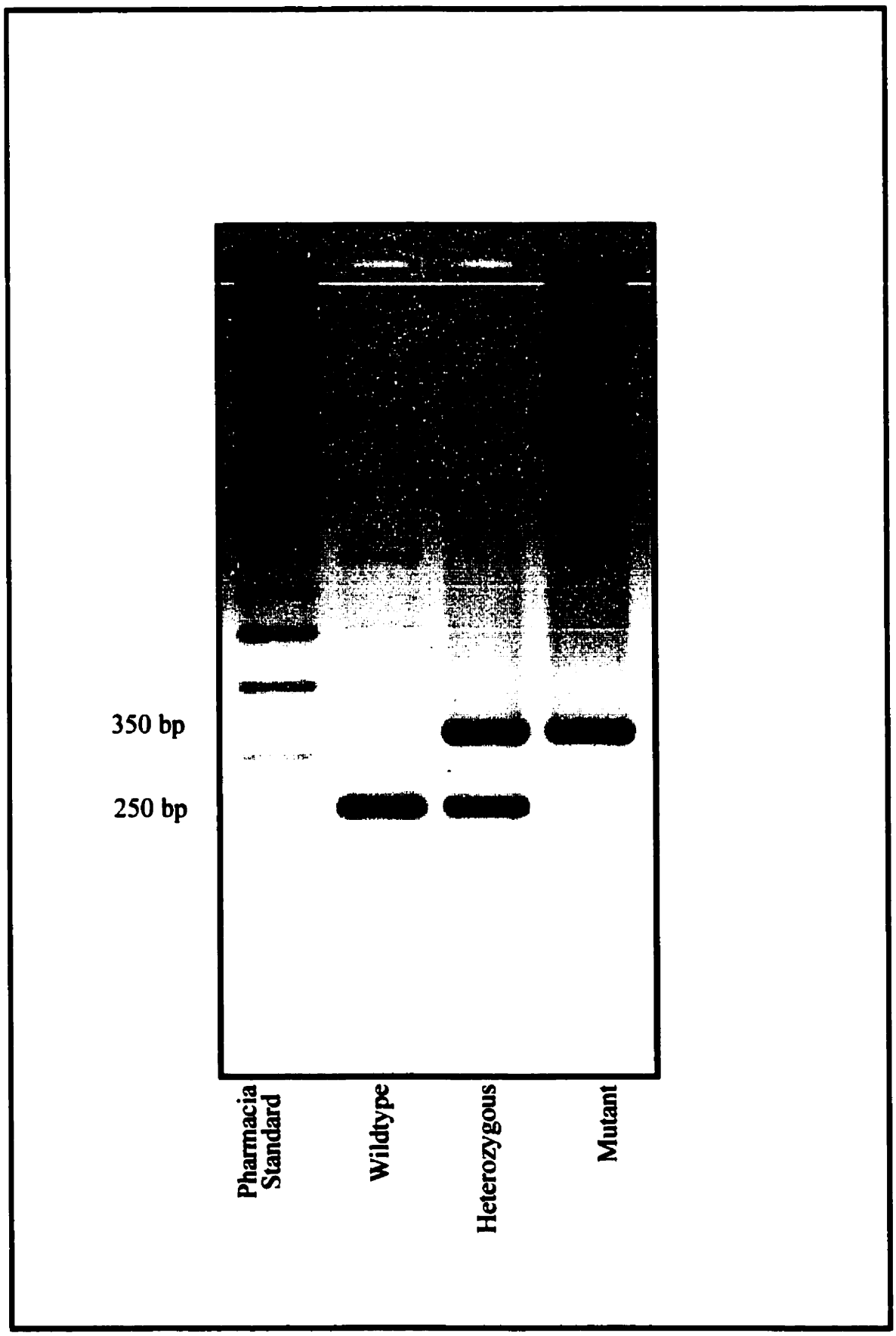

350 bp
250 bp
Pharmacia Standard
Wildtype
Heterozygous
Mutant

Figure 4: Representative radioautographs of cochlear ducts from NT-3 mice at age E19.

Tissue was hybridized with anti-sense probes for FGFR3 mRNA.
Radioactive signal indicated by grains at arrowheads.
A) wildtype, B) mutant, v - vas spirale
Paraffin sections, 5μm thick, modified hematoxylin stain.

Note that to the right of the arrowheads are presumptive "inner" hair cells supporting cells, and the greater epithelial ridges, and to the left are the presumptive "outer" hair cells and supporting cells. The tunnel of Corti will form in the region near the tip of the arrowhead.

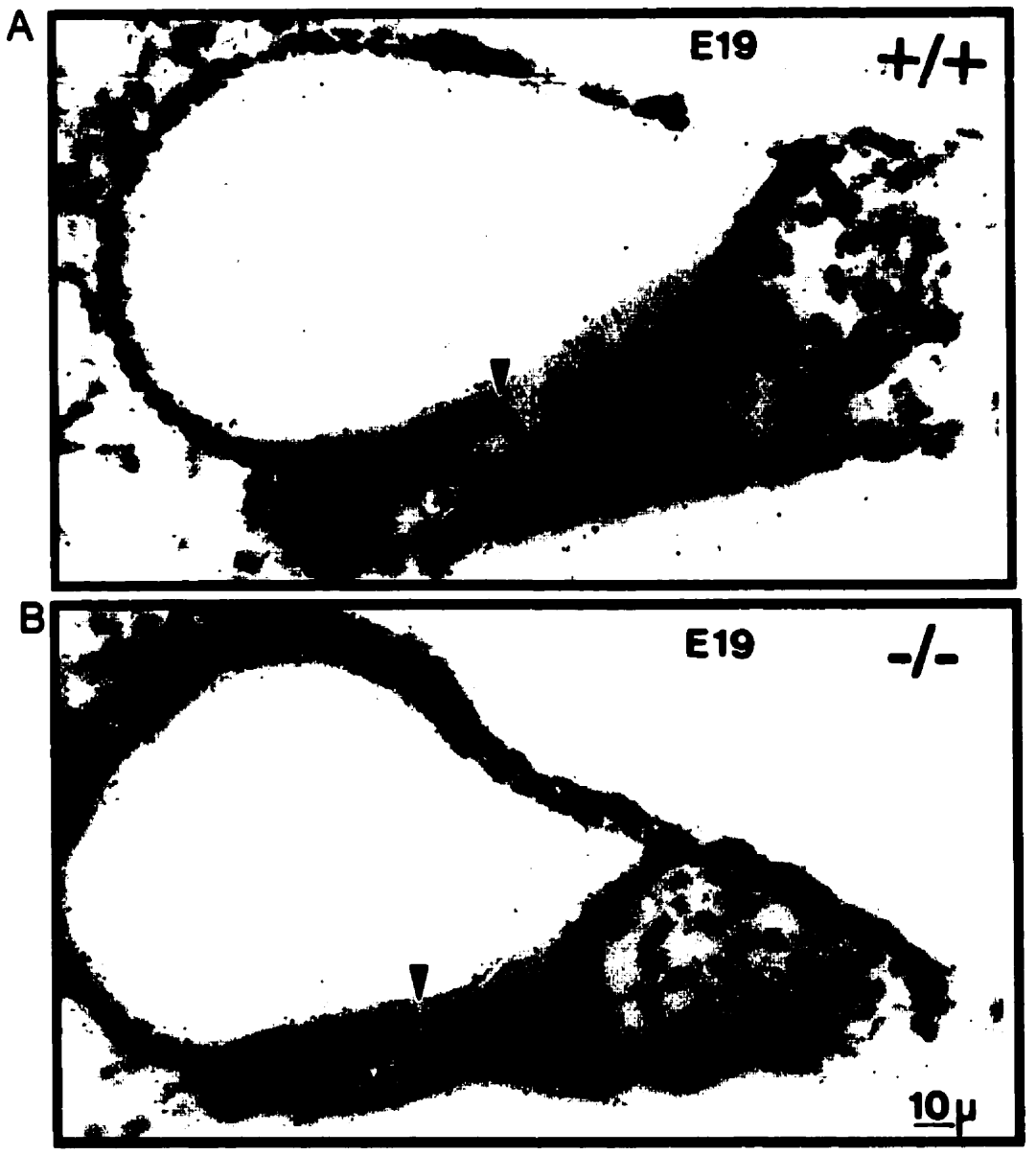

A
E19
+/+
B
E19
-/-
10μ

Figure 5: The developing organ of Corti in age P0 NT-3 mice.
Plastic sections, 3μm thick, H&E stain, high magnification.

A) Heterozygous, 430X magnification, B) Mutant, 400X magnification.

ihc - inner hair cell, ohc - outer hair cell, p - pillar cell, sc - supporting cell, tc - tunnel of Corti, tm - tectorial membrane, vs - vas spirale.

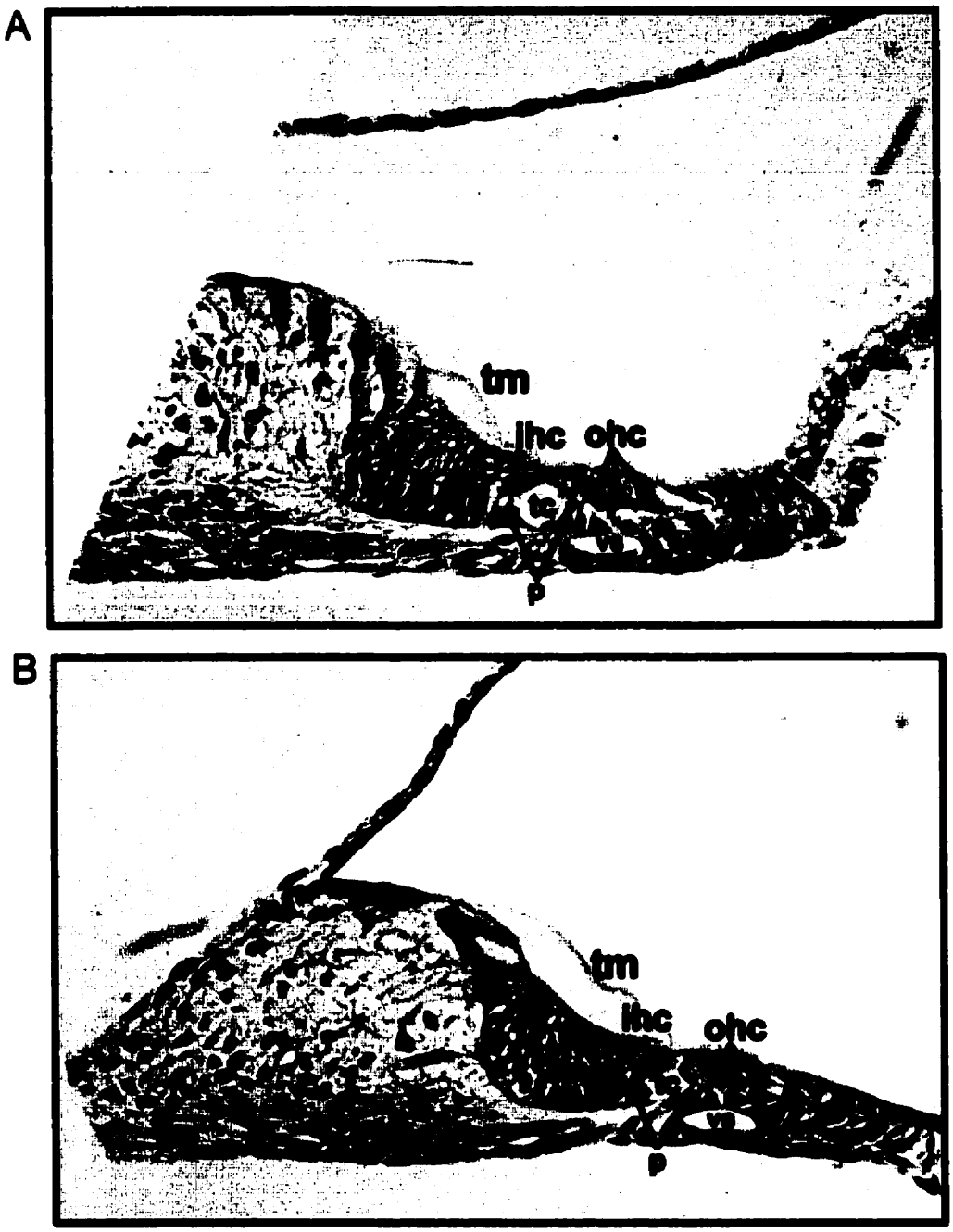

A
tm
ihc
ohc
p
B
tm
ihc
ohc
p

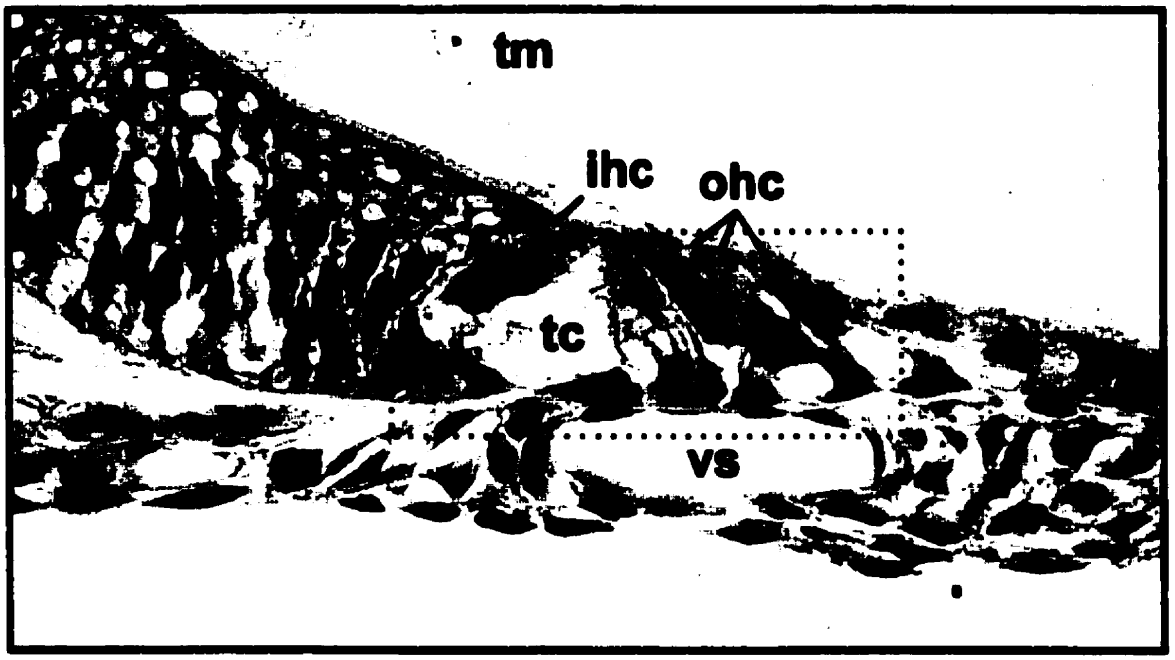
Figure 6*: The developing organ of Corti in age P0 NT-3 mutant mice.
Plastic section, 3μm thick, H&E stain, oil immersion.

A) 1075X magnification, B) 1325X magnification.

ihc - inner hair cell, ohc - outer hair cell, p - pillar cell, sc - supporting cell, tc - tunnel of Corti, tm - tectorial membrane, vs - vas spirale.

*This plate is an enlarged view of part of Figure 5B.

A



B

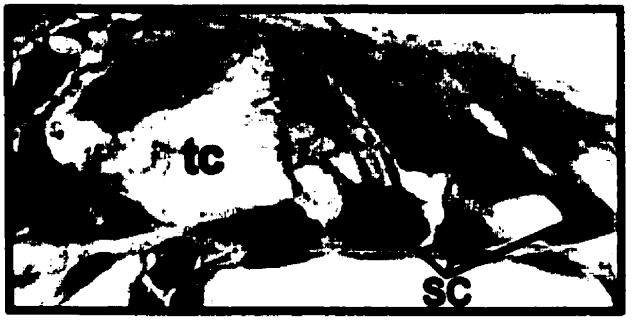


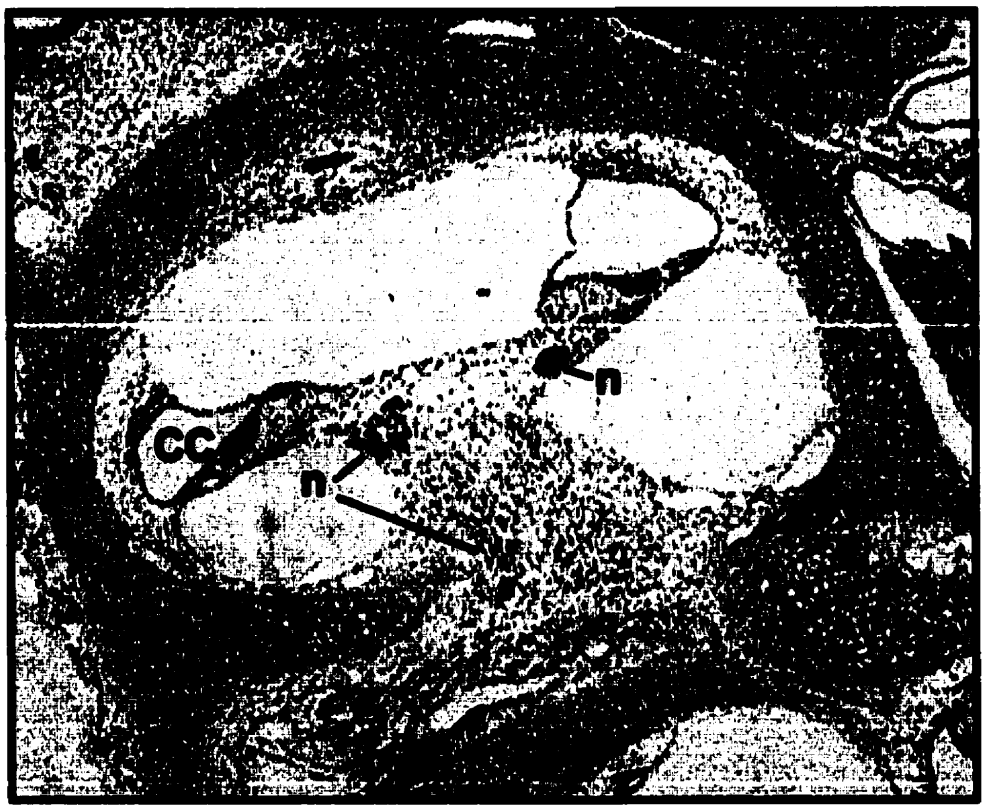
Figure 7: The developing organ of Corti in age P0 NT-3 mice.
Plastic sections, 3μm thick, H&E stain, low magnification.

A) NT-3 heterozygous organ of Corti, note the abundance of neurons (780X magnification).

B) NT-3 mutant organ of Corti; note the apparent reduction of neuronal numbers, particularly in the regions closest to the organ of Corti (550X magnification).

cc - cochlear coil, n - neurons, oc - otic capsule

A



B

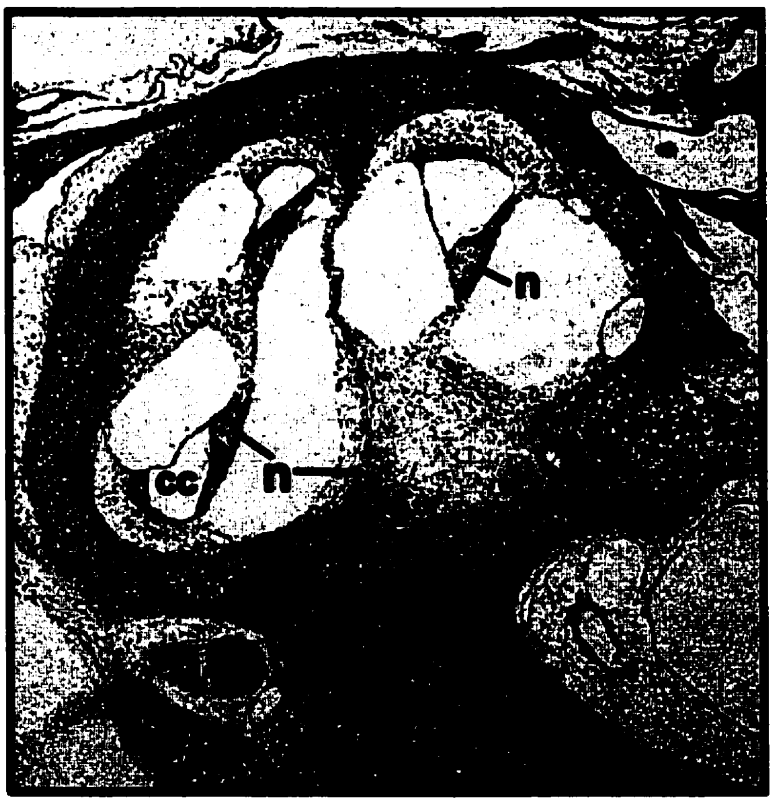


Figure 8: Representative radioautograph of vestibular duct from CD-1 mouse at age E15.5.

Tissue was hybridized with anti-sense probe for FGFR3 mRNA.
Note the absence of reaction in the vestibular system (arrow), and that the otic capsule still has a radioactive signal.
Frozen section, 10μm thick, H&E stain, 90X magnification.

A) brightfield
B) darkfield

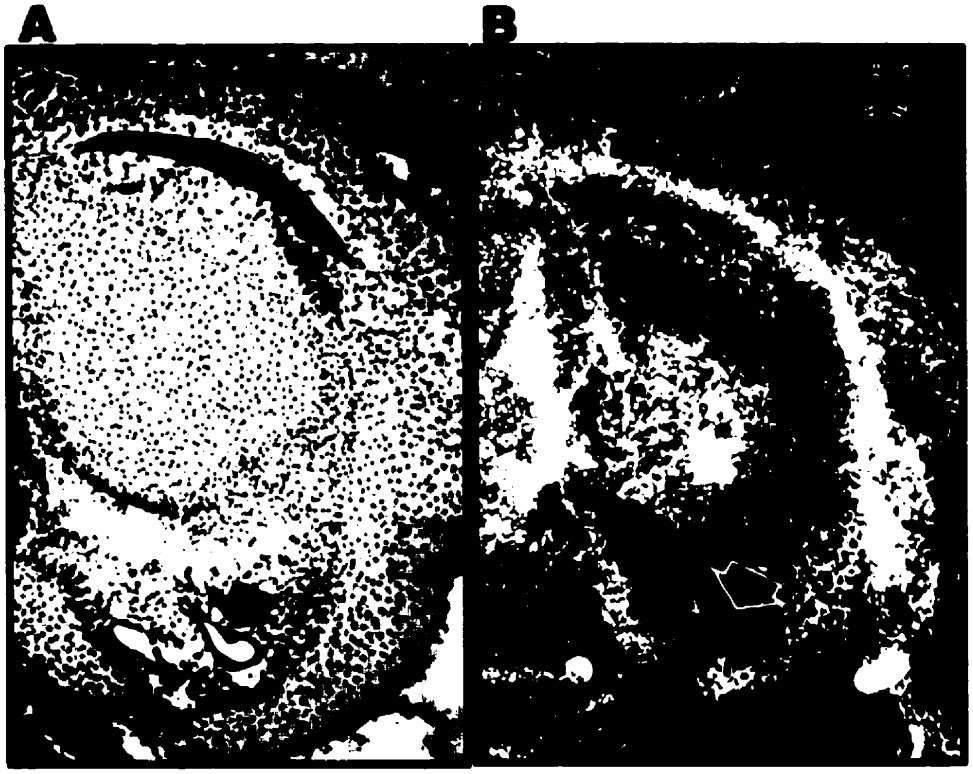

A
B

Figure 9: Representative radioautographs of the organ of Corti in NT-3 mutant mice at age P0.
Tissue was hybridized for FGFR3 mRNA. The right side is inner.
Paraffin sections, 5μm thick, modified hematoxylin stain.

Upper panel - anti-sense probe, radioactive signal at arrowheads; inset, higher magnification of anti-sense reaction (355X magnification, inset 800X magnification).
Lower panel - sense control (355X magnification).

t - tunnel of Corti, v - vas spirale

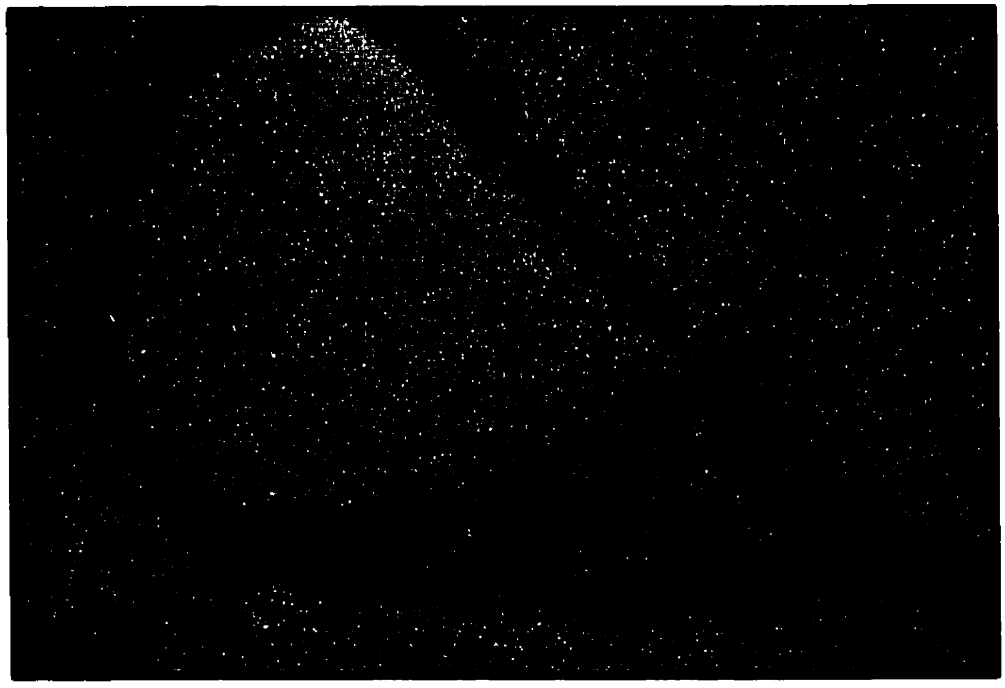
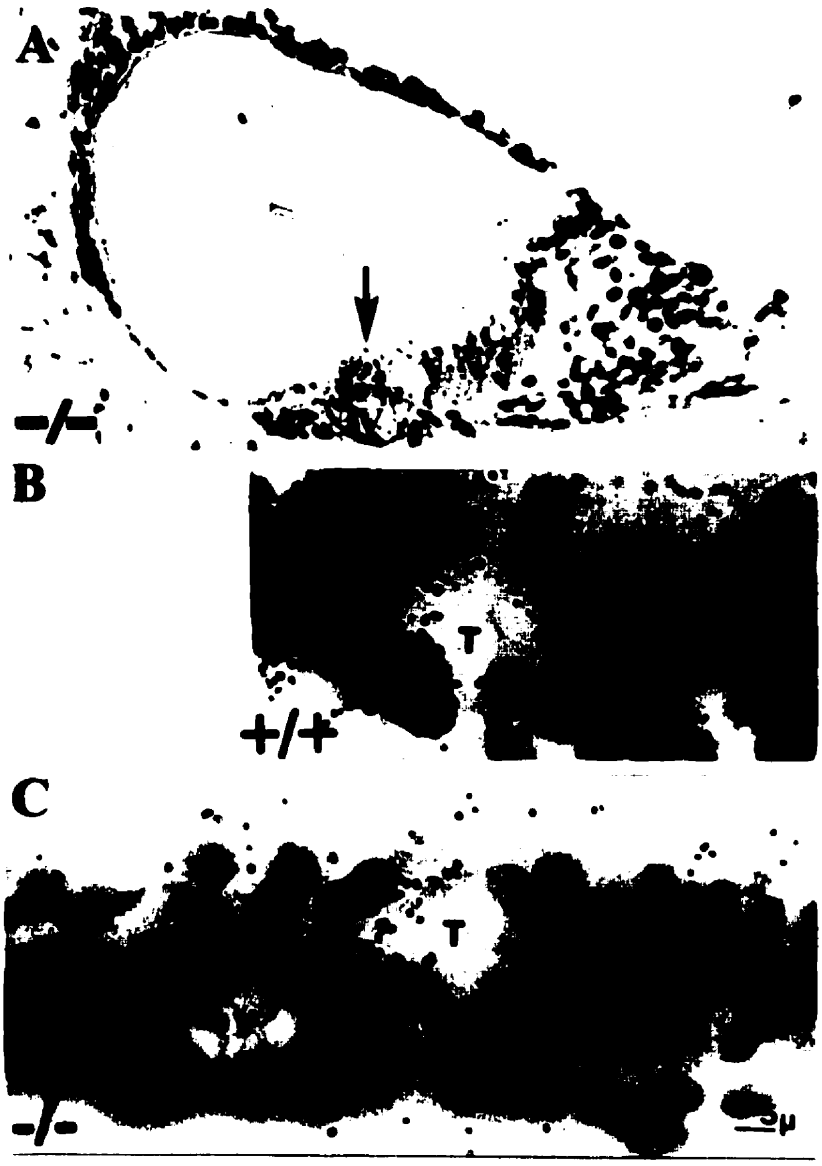


Figure 10: Representative radioautographs of the organ of Corti from age P0 NT-3 mice.
Hybridized with anti-sense probe for FGFR3 mRNA.
The inner side is to the right.
Paraffin sections, 5μm thick, modified hematoxylin stain.

A) Mutant mouse, the radioactive signal at the arrow resembles that found in a wildtype mouse (300x magnification).
B) Wildtype mouse, note grains to the left of the tunnel (T) over the outer cells. Oil immersion.
C) Mutant mouse, note grains to the left of the tunnel (T) over the outer cells. Oil immersion.

T - tunnel of Corti, V - vas spirale


A
-/-
B
T
+/+
C
T
-/-
3μ

Tables

Table 1: Comparison of NT-3 Birth Weights between Genotypes using the Mann-Whitney Rank Sum Test.

	Mutant	Wildtype	Heterozygous
Mean Birth Weight (g)±s.d.	1.202±0.191	1.600±0.298	1.349±0.221
Number of Observations (n)	16	7	10

N=34

The difference between mutant and wildtype medians is statistically significant **(P=0.010)**.

There is insufficient evidence to conclude that there is a difference between mutant and heterozygous medians (P=0.102), and heterozygous and wildtype medians (P=0.079).

Table 2: Comparison of Male and Female NT-3 Birth Weights Within the Same Genotype using the Mann-Whitney Rank Sum Test.

	Mutant		Wildtype		Heterozygous	
	Male	Female	Male	Female	Male	Female
Mean Birth Weight (g)±s.d.	1.197±0.210	1.210±0.174	1.664±0.321	1.440±0.226	1.408±0.189	1.264±0.264
Number of Observations (n)	10	6	5	2	6	4

N=34

Males: Statistical analysis of these data show that the median birth weight of a male mutant mouse is significantly lower that the median birth weight of a male wildtype mouse (P=0.032). There is insufficient evidence to conclude that there is a difference between the median birth weights of male mutant and male heterozygous mice (P=0.074) and male heterozygous and male wildtype mice (P=0.082).

Females: There is insufficient evidence to conclude that there is a difference between the median birth weights of female mutant and female wildtype mice (P=0.429), female mutant and female heterozygous mice (P=1.00), and female heterozygous and female wildtype mice (P=0.533).

Males versus Females of the same genotype: There is insufficient evidence to conclude that there is a difference between the median birth weights of mutant males and females (P=1.00), wildtype males and females (P=0.381), and heterozygous males and females (P=0.352).

Table 3: Comparison of NT-3 Crown-Rump Lengths at Birth between Genotypes using Mann-Whitney Rank Sum Test.

	Mutant	Wildtype	Heterozygous
Mean Birth C-R Length (mm)±s.d.	23.62±4.011	25.88±2.416	24.22±2.386
Number of Observations (n)	13	8	9

There is insufficient evidence to conclude that there is a difference between the crown rump lengths at birth of mutant and wildtype mice (P=0.293), and mutant and heterozygous mice (P=0.973) and heterozygous and wildtype mice (P=0.193).

Table 4: Genotype Percentages Resulting from Matings of NT-3 Heterozygous Mice

Genotype	Percentage of Offspring Observed	Expected Percentage
Wildtype	35.5	25.0
Heterozygous	43.6	50.0
Mutant	20.9	25.0
N = 110	100.0%	100.0%

Table 5: Genotype Percentages Resulting from Matings of NT-3 Heterozygous and Wildtype Mice

Genotype	Percentage of Offspring Observed	Expected Percentage
Wildtype	37.0	50.0
Heterozygous	63.0	50.0
N = 27	100.0%	100.0%

Table 6a: *In situ* Hybridizations at E19 with NT-3 tissue

Genotype	+/+	-/+	-/-
number different animals used	4	2	3
number of experiments	4	4	4
number paraffin slides	4	2	4
number of frozen slides	8	4	10
Total number of slides processed*	**12**	**6**	**14**

Table 6b: *In situ* hybridization at P0 with NT-3 tissue

Genotype	+/+	-/+	-/-
number different animals used	3	5	7
number of experiments	15	15	15
number paraffin slides	10	10	18
number of frozen slides	36	26	44
Total number of slides processed*	**46**	**36**	**62**

*Number of slides actually used in *in situ* hybridization experiments.

Table 7: Localization of FGFR3 mRNA in the Developing Inner Ear of CD-1 Mice

Age	Cochlear Duct	Vestibular Duct	Otic Capsule
E15.5	+	-	+
E17, E19	+	-	+
P0, P3	+	-	+

+ FGFR3 mRNA positive
- FGFR3 mRNA negative

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Appendices

Appendix I

Solutions Guide

1. **Acetylation** (200mL) - 0.1M T.E.A. and 0.25% acetic anhydride
   Note: use fume hood, two 250mL wheaton jars, sterile spin bar, mixer

   Dissolve 3.8g T. E. A. (Triethanolamine, F.W. 185.7, Sigma Chemicals #T-9534) in 200mL DEPC treated water.
   Add 500µL acetic anhydride (F. W. 102.09, Mallincrodt # un1715)to the dry jar.
   Pour the freshly dissolved T.E.A. solution into this boat.
   To boost, add 500µL acetic anhydride to un-used boat, combine with mixing solution.

2. **DEPC Treated Water** (5L) - Diethylpyrocarbonate (Sigma Chemicals #D5758)
   Note: Use fume hood

   Add 5.0mL DEPC to 45mL 100% EtOH.
   Add 50 mL of above solution to 5L of water (5mL in 500mL).
   That is a 0.1% final concentration of DEPC in water
   Slosh the solution around in the bottles.
   Let stand in fume hood overnight.
   Autoclave and store at 4°C

3. **50% Dextran Sulphate** (10mL, Sigma Chemicals #D8906)

   Dissolve 5.0g dextran sulphate in 8.0mL DEPC treated water at 68°C for approximately 3 hours.
   Bring up to a final volume of 10.0mL.
   Use immediately.

4. **DTT** (6.6mL of 5M) - Dithiothreitol, F.W. 154.24, Gibco # 15508-013
   Note: Use fume hood, a sterile flea spin bar and solute can be added directly to the stock bottle of DTT to mix.

   Dissolve 5g DTT in 6.6mL 10mM sodium acetate.
   Filter sterilize using a 0.25µm syringe filter.
   Aliquot in sterile 1.5mL eppendorf tubes in 0.5mL amounts, store at -20°C.

5. EDTA (0.5M, pH 8.0, 1.0L) Anachemia #AC-4231

Dissolve approximately 20.0g NaOH pellets (Fisher # S-315) in 800mL DEPC treated water.
Dissolve 186.1g disodium ethylenediaminetetracetate.$2H_2O$ in this solution.
Note: EDTA does not go into solution until pH reaches 8.0.
Bring to a final volume of 1.0L.
Autoclave and store at room temperature.

6. Formamide Deionization (500mL) Boehringer Manneheim #1814320

Add 5.0g AG501-X8 resin beads (Sigma Chemicals # M-8157) to 50.0mL Falcon tubes.
Add formamide to Falcon tubes.
Shake or stir overnight at 4°C.
Let settle for about 2 hours at 4°C.
Sterilize using a Nalgene 0.45µm vacuum filtration unit.
Store in new 50.0mL Falcon tubes at -20°C.

7. 10xHomogenate Solution (100mL, for PCR)

Dissolve 3.72g KCl (Sigma Chemicals # P-3911) in 81.0mL water and 10.0mL Tris-HCl (1M, pH 8.0, Gibco/BRL # 15568-017).
Adjust pH to 8.3 with 2N NaOH (Fisher # SS264-1).
Add 0.10g gelatin (u.s.p., J.T. Baker Chemical Co. # 1-2124), 4.50mL 10% tween-20 (ICN Biochemicals #) and 4.50mL 10% NP-40 (Sigma Chemicals # P-6507).
Heat gently while stirring to dissolve gelatin.
Store at room temperature for short periods of time.

8. Hybridization Buffer (50.0mL)

Combine in the following order:
25mL deionized formamide
10mL 50% dextran sulphate
3mL 5M NaCl
1mL 50xDenhardts Solution (Sigma Chemicals #D-2532)
0.5mL 1M Tris pH 8.0 (Gibco/BRL #15568-017)
0.1mL 0.5M EDTA (pH 8.0)
0.4mL DEPC teated water

Vortex to mix.
Aliquot in 5mL amounts in 15mL Corning centrifuge tubes and store at -70°C.

9. Hybridization Solution (5.0mL)

*Note: Based on calculations using radioacive probe counts (see #14), will be different for each probe.

Add in the following order to a sterile 15mL corning centrifuge tube:

250 µL tRNA (10mg/mL)
10 µL DTT (5M)
5.5 µL probe*
734.5 µL T.E. buffer (1000 µL - 265.5 µL)
1.0 mL
+ 4.0mL hybridization buffer
5.0mL final volume

Aliquot the 5.0mL of hybridization solution into 500 µL amounts in 1.0mL centrifuge tubes. Freeze at -20°C.

10. Magnesium Chloride (1M, 1L) Anachemica #AC5538

Dissolve 203.3g $MgCl_2.6H_2O$ in 800mL DEPC treated water.
Adjust volume to 1L, autoclave, and store at 4°C.

11. Mouse Tail Solution (40.0mL)

Combine:
1.0mL Tris (2M, pH8)
8.0mL EDTA (0.5M)
0.8mL NaCl (5M)
4.0mL 10% SDS
26.2mL Water (double distilled, deionized)

Store at room temperature.

12. 12% Paraformaldehyde (500mL)

While sirring, heat 500mL PBS to 60°C.
Dissolve 60.0g paraformaldehye (JBS #017) in the warm PBS.
Add a few drops of NaOH (2N, sodium hydroxide, Fisher #SS264-1) to help dissolve powder.
Cool to room temperature.
Filter into an autoclaved bottle using a bottle top Nalgene 0.2 µm PES Tissue Culture Filtration unit (165-0020)
Store at 4°C.

13. **10xPBS** (Phosphate Buffered Saline, pH 7.4, 1L)

Dissolve 87.7g NaCl (Fisher #S640-3)
12.4g Na_2HPO_4 (F.W. 141.96, Mallinckrodt #7917)
1.8g $NaH_2PO_4.H_2O$ (F.W. 137.99, Fisher #S468)
in 600mL DEPC treated water.
Bring to a volume of 2.0L, suction filter, autoclave, store at room temperature.

14. Probe Dilution Calculations

$$\frac{\text{Desired Probe Concentration (CPM/mL)}}{\text{Actual Probe Concentration (CPM/mL)}} = \text{amount of probe (in } \mu\text{L) to be added to } \mathbf{1mL} \text{ of } \mathbf{hybridization\ buffer} \text{ to obtain } \textit{desired probe concentration} \text{ of } \mathbf{hybridization\ solution}$$

5.0mL of hybridization solution is made at one time, so the value calculated is multiplied by 5.

15. **Proteinase K 10mg/mL** stock enzyme (Sigma # P0390)

Dissolve 10mg/mL proteinase K in 0.1M Tris-HCl pH 8.0 (Gibco/BRL #15568-017) and 50mM EDTA.
Store in eppendorf tubes at -20°C.

16. **Proteinase K** working solution (200mL of 0.0001%)

Combine: 160mL DEPC treated water
20mL Tris-HCl (1M, pH 8.0, Gibco/BRL #15568-017)
20mL EDTA (0.5M)
20µL Proteinase K (10mg/mL stock)
Use at 37°C, always make fresh.

17. **RNase A 10mg/mL** stock enzyme (Boehringer Manneheim #109 169)

Dissolve 100mg RNase A in 10mL 0.01M sodium acetate (pH 5.2)
Heat to 100°C for 15 minutes.
PH to 7.7 using 100µL volumes of 1M Tris-HCl (pH 7.4)
Aliquot in eppendorf tubes and store at -20°C.

18. RNase Buffer (1L)

Combine: 10mL Tris-HCl (1M, pH 8.0)
100mL NaCl (5M)
2mL EDTA (0.5M)
888mL water

Adjust to pH 8.0 using a few drops of HCl (1N), store at 4°C.

19. Sodium Acetate (100mM, pH 5.2, 100mL)

Dissolve 1.36g of sodium acetate (F. W. 136.08, Fisher # S209) in 100mL DEPC treated water.
Add approximately 10 drops of glacial acetic acid to adjust pH to 5.2.
Syringe filter using a 0.22µm milipore filter (luer lock, Nalgene #190-2520), autoclave, store at 4°C.

20. Sodium Chloride (NaCl, 5M, 500mL)

Dissolve 146.1g NaCl (Fisher #S640-3) in 500mL DEPC treated water.
Autoclave and store at room temperature.

21. 20xSSC (Standard Saline Citrate, 1L)

Dissolve 175.3g NaCl (Fisher #S640-3) and 88.2g sodium citrate (F. W. 294.10, Anachemia # AC8320) in 900mL DEPC treated water.
Adjust ot pH 7.0 using a few drops of HCl.
Suction filter, autoclave, and store at room temperature.

22. 5xT.B.E. (4L)

Dissolve 216g Tris Base and 110g Boric Acid in 80mL EDTA (0.5M, pH 8.0) and 3920mL Double Distilled De-ionized water.
Store at room temperature.

23. TE Buffer (Tris-EDTA, 1L)

Combine: 10mL Tris-HCl (1M, pH 8.0, Gibco/BRL #15568-017)
2mL EDTA (0.5M)
888mL DEPC treated water

Store at 4°C.

24. Tris-HCl (1M, pH 8.0, 100mL - for when Gibco/BRL runs out)

Dissolve 12.11g of Tris Base (Trizma®, Sigma T-1503) in 95.8mL water and 4.2mL concentrated HCl.

Appendix II

List of Reagents

Reagent	Company	Product Number
Acetic anhydride	Mallinckrodt	un1715
AErrane	OHMEDA	402-223-00
BamHI	Boehringer Manneheim	220612
biopsy cryomolds (10mmX10mmX5mm)	Tissue-Tek	4565
DEPC	Sigma	D5758
50xDenhardt's Solution	Sigma	D2532
dextran sulphate	Sigma	D8906
disposible microtome blades	Fisher	12-631R, No. R35
dNTP's	Pharmacia	27-2035-01
DTT	Gibco	15508-013
EDTA	Anachemia	AC-4231
formamide	Boehringer Manneheim	1814320
gelatin	J.T. Baker Chemical Co.	1-2124
Harris hematoxylin (modified) (no acetic acid, no mercury)	Fisher	SH30-500D
0.2N HCl	Anachemia	R-2830D
isopentane	ACROS Organics	12647-0010
KCl	Sigma	P3911
Kodak D-19	Don's Photo	146 4593
Kodak Fixer	Don's Photo	123 8146
Kodak NTB-2	Interscience	1654433
Magnesium Chloride	Anachemia	AC-5538

Reagent	Company	Product Number
NaCl	Fisher	S640-3
NaOH pellets	Fisher	S-315
2N NaOH	Fisher	SS264-1
Na_2HPO_4	Mallinckrodt	7917
$NaH_2PO_4 \cdot H_2O$	Fisher	S468
NP-40	Sigma	P6507
OCT compound	Tissue-Tek	4583
paraformaldehyde	JBS	017
Polylysine microscope slides	Esco	P-4981
proteinase K	Sigma	P0390
RNase A	Boehringer Manneheim	109169
sucrose	Mallinckrodt	AR8360
sodium acetate	Fisher	S209
sodium citrate	Anachemia	AC8320
SP6	Boehringer Manneheim	810274
Superfrost Plus microscope slides	Fisher	12-550-15
T7	Boehringer Manneheim	881767
Taq polymerase	Pharmacia	27-0799-01
TEA	Sigma	T9534
tris-HCl (pH 8.0)	Gibco/BRL	15568-017
tris (Trizma base®)	Sigma	T-1503
tween-20	ICN Biochemicals	194841
UTP	ICN Parmaceuticals Inc.	56103H
XbaI	Boehringer Manneheim	67257

Appendix III

_____ timed mating

DAM: birthday_____1996
colour: white brown-grey
identific.tag # 39_____-/+
prev litt?

SIRE: birthday_____1996
colour: white brown-grey
idetific.tag # 39_____-/+
prev.litt?

DATE of BREEDING: 1997
Date confirmed pregnant: Plug seen on:
Date and time of delivery:
Number of young:
Gender/phenotye/genotype/identification of young:

gender									
colour /eye pig									
milk?									
wgt									
CR									
tag#									
geno									
blood									
tail									
other									

NOTES:

Appendix IV

PCR Primer Sequences for NT-3 Mouse Genotyping

oIMR130 5'-CCT GGC TTC TTT ACA TCT CG-3'

oIMR131 5'-TGG AGG ATT ATG TGG GCA AC-3'

oIMR132 5'-GGG AAC TTC CTG ACT AGG GG-3'

Primer sequences supplied by *The Jackson Laboratory* web page (http://lena.jax.org/re...protocols/Ntf3_KO.html), as per Dr. Patrik Ernfors' (Karolinski Institute, Stockholm Sweden) original protocol.