Anatomical Studies of the Olivocochlear Collaterals System

Ahmaed Baashar

Supervisors: Associate Professor: Wilhelmina Mulders
Winthrop Professor: Donald Robertson

This thesis is presented for the degree of Doctor of Philosophy

THE UNIVERSITY OF WESTERN AUSTRALIA
School of Human Sciences
Faculty of Life and Physical Sciences
Crawley WA 6009, Australia
2017
Abstract

The auditory system, like the other sensory systems, consists of afferent and efferent neural pathways. One part of the efferent pathways is the medial Olivocochlear (MOC) pathway, which is thought to be involved in discrimination and detection of complex signals in noisy environments, protecting the cochlea from damage during intense acoustic exposure, attention-focused sound detection and regulation of endocochlear potential (EP) in the cochlea (Scharf et al., 1994, Scharf et al., 1997, Rajan, 2000, Patuzzi, 2002, Guinan, 2006, 2011).

The MOC pathway arises from cells located in the superior olivary complex (SOC) and terminates on the cochlear outer hair cells (OHCs) (Warr and Guinan, 1979, Robertson, 1985). The MOC pathway also sends out collateral branches that terminate in the first brainstem nucleus of the afferent pathway, the cochlear nucleus (CN). These collateral branches have been shown to make synaptic contact with multipolar cells (Brown et al., 1988, Brown et al., 1991, Brown and Benson, 1992, Benson et al., 1996). Two distinct classes of multipolar cells have been identified in the CN: (i) T-stellate cells which are thought to project to the inferior colliculus (IC), and (ii) D-stellate cells which are thought to project to the contralateral CN. These classes of multipolar cells play distinct functional roles in neuronal information processing. Therefore, it is important to determine the targets of the MOC collateral branches in the CN.

Controversy exists in current literature as to which multipolar cells are the targets of MOC pathway, the T-stellate cells or D-stellate cells (Fujino and Oertel, 2001, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). Therefore, the main objective of this thesis was to study the anatomical relationship between CN multipolar cells projecting to the IC or to the contralateral CN (thought to represent T-stellate cells and D-stellate cells, respectively) and the collateral branches of MOC neurons. In the first part of the study (Chapters 3, 4 and 5), the retrograde tracer Fluorogold (FG) was injected into the IC or CN of guinea pigs and rats to label the T-stellate cells or D-stellate cells, respectively. The collateral branches of MOC neurons were then labelled by injection of biocytin at the centre of the floor of the fourth ventricle, where MOC axons are grouped in a tight bundle between the facial genua. In the second part of the study (Chapter 6), the neuronal tracer biocytin was injected at the
centre of the floor of the fourth ventricle in transgenic mice, which express enhanced green fluorescent protein (EGFP) under the control of the promotor of the glycine transporter 2 (GlyT2) gene, to assess whether glycinergic cells (presumably including D-stellate cells) receive synaptic innervation from the collateral branches of MOC neurons (Altschuler et al., 1986, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Friauf et al., 1999, Zeilhofer et al., 2005). Close apposition of retrogradely labelled cells (guinea pigs and rats) or glycinergic cells (mice) and MOC axonal varicosities were evaluated as a putative synaptic contact.

Putative synaptic contacts between the anterogradely labelled MOC axonal varicosities and retrogradely labelled T-stellate cells and D-stellate cells were found, but only in very low numbers. In guinea pigs, only 0.28% of the labelled MOC axonal varicosities made putative synaptic contact with the T-stellate cells, but none of the labelled MOC axonal varicosities were seen to make contact with D-stellate cells. In rats, 0.22% of the labelled MOC axonal varicosities made putative synaptic contact with T-stellate cells and 0.22% made putative synaptic contact with D-stellate cells. No evidence was found for synapses between glycinergic (D-stellate) cells and MOC axonal varicosities in the CN of transgenic mice. In all successfully labelled guinea pigs, rats and transgenic mice, the anterogradely labelled MOC axonal varicosities were observed to make putative contact with many unlabelled CN cells. There were some variations in the distribution pattern of MOC collateral branches and retrogradely labelled cells in the PVCN and AVCN in both the guinea pigs and rats. Axonal varicosities of MOC neurons in most guinea pigs were found in the core of the VCN, whilst they were located mainly on the medial edge of the VCN in all rats. The total number of the FG labelled multipolar cells projecting to the IC or to the contralateral CN in guinea pigs was less than those observed in rats.

The findings of this thesis do not provide compelling evidence that the MOC collaterals provide significant direct input to either T-stellate cells or D-stellate cells, as defined either by their axonal trajectories or glycinergic nature. These results suggest possibly that T-stellate cells and D-stellate cells may not be well defined by their projections or that the suggested relationship between anatomical and electrophysiological characteristics (i.e. T-stellate corresponding to chopper and D-stellate to onset chopper cells) may not be strictly correct. The results are possibly consistent with the notion that most MOC collateral
branches innervate other classes of multipolar or non-multipolar CN cells that remain to be identified.
Acknowledgments

I would like to thank several people for their personal contribution to my studies and completion of my PhD thesis.

First of all, I would like to thank my principal supervisor Winthrop Professor Donald Robertson for his constant encouragement, support and guidance throughout my candidature.

Importantly, I would like to offer my sincere thanks to my co-supervisor Associate Professor Wilhelmina Mulders for her unrelenting patience and willingness to assist despite her busy schedule.

Many thanks to the administrative, academic staff and students in the School of Human Science, particularly in the Auditory Laboratory, for providing an accommodating and encouraging study environment.

Much appreciation goes to the University of King Saud bin Abdulaziz for Health Sciences for providing me the scholarship. The financial support provided by this scholarship allowed me to focus on my studies throughout the course.

Endless thanks and appreciated to my mum, dad, brothers and sisters for their unconditional support and source of strength.

Finally, my deepest thanks go to my wife (Dr. Nada Townsi) and son (Saden) for their exceptional patience and encouragement, I love you both and I would not have been able to do it without your support.
Statement of Candidate Contribution

I certify that the work contained in this thesis is an account of my research undertaken during the period of 25th August 2012 to 25th July 2017 while I was enrolled as a full-time postgraduate student in the Discipline of Neuroanatomy, School of Human Sciences at the University of Western Australia. Except for occasional assistance in the experimental set-up from my supervisors (Winthrop Professor Donald Robertson and Associate Professor Wilhelmina Mulders), all experimental were performed by me. The content of this thesis is my own composition, and all relevant sources are acknowledged. This thesis has never been accepted for any other degree in this or another institution.

Ahmaed Baashar
25th of July 2017
Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgments ......................................................................................................................... v
Statement of Candidate Contribution ........................................................................................... vi
List of Figures .................................................................................................................................. xi
List of Tables ................................................................................................................................... xvii
List of Abbreviations .................................................................................................................... xix

Chapter 1 Literature Review and General Introduction .............................................................. 1
  1.1. General Overview .................................................................................................................. 1
  1.2. Anatomy of the Cochlear Nucleus ....................................................................................... 2
      1.2.1. Fibre Tracts of the Cochlear Nucleus ........................................................................ 3
      1.2.2. Cytoarchitectural Regions of the Cochlear Nucleus ................................................... 4
  1.3. The Olivocochlear Efferent System ..................................................................................... 13
      1.3.1. Physiology of the Olivocochlear Efferent System ...................................................... 13
  1.4. Anatomy of the Olivocochlear Efferent System ................................................................. 25
      1.4.1. Pathways of Olivocochlear Efferent Neurons ............................................................ 27
      1.4.2. Collateral Branches of Olivocochlear Efferent Neurons into the Cochlear Nucleus .... 31
      1.4.3. Targets of Medial Olivocochlear Collateral Branches in the Cochlear Nucleus ......... 32
  1.5. Potential Role of the Medial Olivocochlear Collateral Branches ....................................... 36
      1.5.1. Signal Detection in Noise ............................................................................................ 36
      1.5.2. Central Detection Adjustment ..................................................................................... 36
      1.5.3. Modulating the Neural Hyperactivity in the Central Auditory Pathways .................. 36
  1.6. The Specific Aims of the Thesis ............................................................................................ 38
# Chapter 2 General Methods

2.1. Overview of Experimental Design in Guinea Pigs and Rats ............................................. 39

2.2. Overview of Experimental Design in Mice ....................................................................... 39

2.3. Animal Subjects .............................................................................................................. 41

2.4. Retrograde Tracer Injections in Guinea Pigs and Rats ...................................................... 41

2.4.1. Anaesthesia ................................................................................................................ 41

2.4.2. Surgical Procedures .................................................................................................. 41

2.4.3. Neural Recording and Stimulus Generation and Presentation in Guinea Pigs only..... 42

2.4.4. Fluorogold Tracer Injections ..................................................................................... 43

2.5. Bidirectional Tracer Injections in Guinea Pigs, Rats and Mice .......................................... 44

2.5.1. Anaesthesia ................................................................................................................ 44

2.5.2. Electrical Stimulation of Facial Nerve and Biocytin Tracer Injections ....................... 44

2.6. Histological Processing ................................................................................................ 45

2.7. Histological Analysis ...................................................................................................... 49

# Chapter 3 Selective Labelling of MOC Neurons and their Collateral Branches in the Cochlear Nucleus in the Guinea Pig, Rat and Mouse .............................................................................. 60

3.1. Introduction ...................................................................................................................... 60

3.2. Results ........................................................................................................................... 60

3.2.1. Locations and Characteristics of Biocytin Injection Sites ........................................... 60

3.2.2. MOC Axons and their Collateral Branches in the Cochlear Nucleus .......................... 69

3.3. Discussion ....................................................................................................................... 81

# Chapter 4 Anatomical Relationship between Multipolar (T-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat .................................................. 85
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>85</td>
</tr>
<tr>
<td>4.2</td>
<td>Results</td>
<td>85</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Locations and Characteristics of FG Injection Sites</td>
<td>85</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Distribution of Labelled cells in the Nuclei of the Auditory System</td>
<td>88</td>
</tr>
<tr>
<td>4.2.3</td>
<td>MOC Axonal Varicosities and Multipolar Cells in the Ventral Cochlear Nucleus</td>
<td>94</td>
</tr>
<tr>
<td>4.3</td>
<td>Discussion</td>
<td>99</td>
</tr>
<tr>
<td>5.1</td>
<td>Anatomical Relationship between Multipolar (D-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat</td>
<td>105</td>
</tr>
<tr>
<td>5.2</td>
<td>Results</td>
<td>105</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Locations and Characteristics of FG Injection Sites</td>
<td>105</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Distribution of Labelled Cells in the Nuclei of the Auditory System</td>
<td>108</td>
</tr>
<tr>
<td>5.2.3</td>
<td>MOC Axonal Varicosities and Multipolar Cells in the Ventral Cochlear Nucleus</td>
<td>115</td>
</tr>
<tr>
<td>5.3</td>
<td>Discussion</td>
<td>118</td>
</tr>
<tr>
<td>6.1</td>
<td>Anatomical Relationship between Glycinergic Cells and MOC Collateral Branches in the Cochlear Nucleus of Transgenic Mice</td>
<td>124</td>
</tr>
<tr>
<td>6.2</td>
<td>Results</td>
<td>124</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Distribution of Labelled Cells in the Nuclei of the Auditory System</td>
<td>124</td>
</tr>
<tr>
<td>6.2.2</td>
<td>MOC Axonal Varicosities and Glycinergic Cells in the Ventral Cochlear Nucleus</td>
<td>131</td>
</tr>
<tr>
<td>6.3</td>
<td>Discussion</td>
<td>135</td>
</tr>
<tr>
<td>7.1</td>
<td>General Discussion and Conclusions</td>
<td>140</td>
</tr>
<tr>
<td>7.1</td>
<td>Aims of the Thesis</td>
<td>140</td>
</tr>
</tbody>
</table>
7.2. Summary of Findings ........................................................................................................140
7.3. Technical Considerations ..............................................................................................140
7.4. Interpretation and Implications ......................................................................................143
7.5. Suggestions for Future Research ..................................................................................146
7.6. Conclusion ....................................................................................................................147

References ..........................................................................................................................148
List of Figures

Chapter 1: Literature Review and General Introduction

Figure 1-1: Anatomical location of the cochlear nucleus and adjacent structures.................................3
Figure 1-2: Distribution of cell types and fibre tracts in all subdivisions of the cochlear nucleus .......5
Figure 1-3: Medial olivocochlear fibres synapse on outer hair cells ..................................................15
Figure 1-4: Schematic representation of medial olivocochlear stimulation on input-output functions of primary afferents responses to characteristic frequency tone bursts in quiet and noisy backgrounds.................................................................................................................................19
Figure 1-5: Origin, course and termination of the olivocochlear neurons .........................................29

Chapter 2: General Methods

Figure 2-1: Schematic representation of experimental set-up.................................................................40
Figure 2-2: Photomicrographs of retrograde labelling in the cochlear nucleus in guinea pigs using immuno-labelling methods.................................................................................................................................48
Figure 2-3: A series of toluidine blue-counterstained transverse sections illustrating the location of the cytoarchitectonic borders of the cochlear nucleus in guinea pig.............................................50
Figure 2-4: A series of toluidine blue-counterstained transverse sections illustrating the location of the cytoarchitectonic borders of the cochlear nucleus in rat.........................................................51
Figure 2-5: A series of toluidine blue-counterstained transverse sections illustrating the location of the cytoarchitectonic borders of the cochlear nucleus in mouse .................................................52
Figure 2-6: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of the ventral nucleus of the trapezoid body, the dorsomedial periolivary nucleus and the rostral periolivary nucleus in guinea pig .................................................................53
Figure 2-7: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of the ventral nucleus of the trapezoid body and the rostral periolivary nucleus in rat........................................................................................................54
Figure 2-8: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of the ventral nucleus of the trapezoid body and the rostral periolivary nucleus in mouse .................................................................55

Figure 2-9: Photomicrographs showing FG labelled cells in the ventral cochlear nucleus........56

Figure 2-10: Photomicrographs showing an example of putative close apposition of an FG labelled cell and medial olivocochlear axonal varicosity.................................................................58

Figure 2-11: Photomicrographs showing a misleading example of putative close apposition of an FG labelled cell and medial olivocochlear axonal varicosity.................................................................59

Chapter 3: Selective Labelling of MOC Neurons and their Collateral Branches in the Cochlear Nucleus in the Guinea Pig, Rat and Mouse

Figure 3-1: Photomicrographs showing the results of successful and unsuccessful injections in guinea pig.........................................................................................................................................................61

Figure 3-2: Photomicrographs showing the results of successful and unsuccessful injections in rat..62

Figure 3-3: Photomicrographs showing the results of successful and unsuccessful injections in mouse ......................................................................................................................................................63

Figure 3-4: Photomicrographs showing the absence of retrogradely labelled cells in the dorsal cochlear nucleus following successful injections of the tracer biocytin at the centre of the floor of the fourth ventricle in the guinea pigs, rats and the mouse.................................................................64

Figure 3-5: Counts of medial olivocochlear neurons in the ventral nucleus of the trapezoid body, the rostral periolivary nucleus and the dorsomedial periolivary nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs…68

Figure 3-6: Counts of medial olivocochlear neurons in the ventral nucleus of the trapezoid body and the rostral periolivary nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats........................................................................................................68

Figure 3-7: Photomicrographs showing the locations of labelled medial olivocochlear collateral branches in the guinea pigs ...........................................................................................................................................70
Figure 3-8: Photomicrographs showing the locations of labelled medial olivocochlear collateral branches in the rats ................................................................. 71

Figure 3-9: Photomicrographs showing the locations of labelled medial olivocochlear collateral branches in the mouse ................................................................ 72

Figure 3-10: A series of drawings of transverse sections of the dorsal and ventral cochlear nuclei demonstrating the locations of medial olivocochlear collateral branches in the granule cell layer, the dorsal cochlear nucleus and medial edge and core of the ventral cochlear nucleus following biocytin injections at the centre of the floor of the fourth ventricle in the guinea pigs ........................................ 73

Figure 3-11: A series of drawings of transverse sections of the dorsal and ventral cochlear nuclei demonstrating the locations of medial olivocochlear collateral branches in the granule cell layer, the dorsal cochlear nucleus and medial edge and core of the ventral cochlear nucleus following biocytin injections at the centre of the floor of the fourth ventricle in the rats .................................................. 74

Figure 3-12: A series of drawings of transverse sections of the dorsal and ventral cochlear nuclei demonstrating the locations of medial olivocochlear collateral branches in the granule cell layer, the dorsal cochlear nucleus and medial edge and core of the ventral cochlear nucleus following biocytin injections at the centre of the floor of the fourth ventricle in the mouse ........................................ 75

Figure 3-13: High magnification images of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs, rats and the mouse ......................... 76

Figure 3-14: Counts of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs .............................................................................. 79

Figure 3-15: Counts of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats ......................................................................................... 79

Figure 3-16: An apparent relationship exists between the total number of the biocytin-labelled medial olivocochlear neurons and the total number of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem in the guinea pigs and rats ....................................................................................................................................... 81
Chapter 4: Anatomical Relationship between Multipolar (T-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat

Figure 4-1: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the inferior colliculus in guinea pigs.................................................................86

Figure 4-2: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the inferior colliculus in rats.................................................................87

Figure 4-3: Photomicrographs of retrograde labelling in the cochlear nucleus after a unilateral injection of FG into the inferior colliculus in guinea pigs...........................................................................90

Figure 4-4: Photomicrographs of retrograde labelling in the cochlear nucleus after a unilateral injection of FG into the inferior colliculus in rats......................................................................................91

Figure 4-5: A series of drawings of transverse sections of the contralateral dorsal and ventral cochlear nuclei showing the locations of labelled cells following the retrograde tracer FG injections into the central region of the inferior colliculus in guinea pig .................................................................93

Figure 4-6: A series of drawings of transverse sections of the contralateral dorsal and ventral cochlear nuclei showing the locations of labelled cells following the retrograde tracer FG injections into the ventral region of the inferior colliculus in rat into the ventral region of the inferior colliculus in rat ........................................................................................................................................93

Figure 4-7: A series of drawings of transverse sections of the contralateral dorsal and ventral cochlear nuclei showing the locations of labelled cells following the retrograde tracer FG injections into the dorsal region of the inferior colliculus in rat......................................................................................94

Figure 4-8: Counts of FG and biocytin-labelled cells following successful injections into the inferior colliculus and at the floor of fourth ventricle in four guinea pigs ..........................................................97

Figure 4-9: Counts of FG and biocytin-labelled cells following successful injections into the inferior colliculus and at the floor of fourth ventricle in five rats ..................................................................................97

Figure 4-10: Light microscopic image showing synaptic contacts between biocytin-labelled medial olivocochlear axonal varicosities and FG labelled multipolar (T-stellate) cells in the ventral cochlear nucleus of guinea pigs and rats ........................................................................................................................................98
Figure 4-11: Photomicrographs showing examples of putative synaptic contacts between medial olivocochlear axonal varicosities and unlabelled ventral cochlear nucleus structures in guinea pigs and rats .................................................................99

Chapter 5: Anatomical Relationship between Multipolar (D-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat

Figure 5-1: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the ventral cochlear nucleus in guinea pigs.................................106

Figure 5-2: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the ventral cochlear nucleus in rats.........................................................107

Figure 5-3: Numbers of FG labelled cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the guinea pigs...110

Figure 5-4: Numbers of FG labelled cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the rats........110

Figure 5-5: A series of drawings of transverse sections of the contralateral dorsal and ventral cochlear nuclei showing the location of labelled cells following the retrograde tracer FG injections into the ventral cochlear nucleus in guinea pig.................................................................112

Figure 5-6: A series of drawings of transverse sections of the contralateral dorsal and ventral cochlear nuclei showing the location of labelled cells following the retrograde tracer FG injections into the ventral cochlear nucleus in rat.................................................................113

Figure 5-7: The cell body areas of the retrogradely labelled multipolar cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the guinea pigs.................................................................114

Figure 5-8: The cell body areas of the retrogradely labelled multipolar cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the rats.................................................................114

Figure 5-9: Light microscope showing synaptic contacts between biocytin-labelled medial olivocochlear axonal varicosities and FG labelled multipolar (D-stellate) cells in the ventral cochlear nucleus in rats.................................................................117
Figure 5-10: Photomicrographs showing examples of putative synaptic contacts between medial olivocochlear axonal varicosities and unlabelled ventral cochlear nucleus structures in guinea pigs and rats .......................................................... 118

Chapter 6: Anatomical Relationship between Glycinergic Cells and MOC Collateral Branches in the Cochlear Nucleus of Transgenic Mice

Figure 6-1: Photomicrograph of EGFP labelled cells in some nuclei of the superior olivary complex in transgenic mice ................................................................. 126

Figure 6-2: Photomicrographs of EGFP labelled cells in the dorsal cochlear nucleus, the posterior ventral cochlear nucleus and the anterior ventral cochlear nucleus in transgenic mice .................. 127

Figure 6-3: Photomicrographs of EGFP labelled cells in the dorsal cochlear nucleus, the posterior ventral cochlear nucleus and the anterior ventral cochlear nucleus in transgenic mice .......... 128

Figure 6-4: A series of drawings of transverse sections of the dorsal and ventral cochlear nuclei showing the location of labelled large and small cells in the posterior and anterior ventral cochlear nuclei in a transgenic mouse ................................................................. 130

Figure 6-5: A series of photomicrographs showing the absence of putative synaptic contact between the EGFP labelled cells and medial olivocochlear axonal varicosities in the ventral cochlear nucleus in a transgenic mouse ................................................................. 133

Figure 6-6: A series of photomicrographs showing the absence of putative synaptic contact between the EGFP labelled cell and medial olivocochlear axonal varicosities in the ventral cochlear nucleus in a transgenic mouse ................................................................. 134
List of Tables

Chapter 3: Selective Labelling of MOC Neurons and their Collateral Branches in the Cochlear Nucleus in the Guinea Pig, Rat and Mouse

Table 3-1: Numbers of biocytin-labelled medial olivocochlear neurons in the ventral nucleus of the trapezoid body, the rostral periolivary nucleus and the dorsomedial periolivary nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs..........................................................66

Table 3-2: Numbers of biocytin-labelled medial olivocochlear neurons in the ventral nucleus of the trapezoid body and the rostral periolivary nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats...............................67

Table 3-3: Numbers of biocytin-labelled medial olivocochlear neurons in the ventral nucleus of the trapezoid body and the rostral periolivary nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the mouse.........................69

Table 3-4: Numbers of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs..........................................................77

Table 3-5: Numbers of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats.................................................................78

Table 3-6: Numbers of medial olivocochlear en passant and terminal varicosities in the ventral cochlear nucleus on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the mouse .................................................................80

Chapter 4: Anatomical Relationship between Multipolar (T-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat

Table 4-1: Numbers of FG and biocytin-labelled cells and medial olivocochlear axonal varicosity in the ventral cochlear nucleus contralateral to the FG injection site in the guinea pigs.........................95
Table 4-2: Numbers of FG and biocytin-labelled cells and medial olivocochlear axonal varicosity in the ventral cochlear nucleus contralateral to the FG injection site in the rats .......................................................... 95

Table 4-3: Numbers and location of putative synaptic contacts in the ventral cochlear nucleus contralateral to the FG injection site in the guinea pigs ................................................................. 96

Table 4-4: Numbers and location of putative synaptic contacts in the ventral cochlear nucleus contralateral to the FG injection site in the rats ................................................................. 96

**Chapter 5: Anatomical Relationship between Multipolar (D-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat**

Table 5-1: Numbers of FG and biocytin-labelled cells and medial olivocochlear axonal varicosity in the ventral cochlear nucleus contralateral to the FG injection site in the guinea pigs ...................... 115

Table 5-2: Numbers of FG and biocytin-labelled cells and medial olivocochlear axonal varicosity in the ventral cochlear nucleus contralateral to the FG injection site in the rats .............................. 115

Table 5-3: Numbers and locations of putative synaptic contacts in the ventral cochlear nucleus contralateral to the FG injection site in the guinea pigs .............................................................. 116

Table 5-4: Numbers and locations of putative synaptic contacts in the ventral cochlear nucleus contralateral to the FG injection site in the rats ........................................................................ 116
List of Abbreviations

Units

- dB SPL  Decibels Sound Pressure Level
- kHz     Kilohertz
- mm      Millimetre
- ms      Millisecond
- mV      Millivolt
- nm      Nanometre

Terms

- ABER    Auditory Brainstem Evoked Response
- AC      Auditory Cortex
- Ach     Acetylcholine
- AChE    Acetylcholinesterase
- ARC     Animal Resource Centre
- AVCN    Anterior Ventral Cochlear Nucleus
- BSA     Bovine Serum Albumin
- CAP     Compound Action Potential
- CGRP    Calcitonin Gene-Related Peptide
- ChAT    Choline Acetyltransferase
- CM      Cochlear Microphonic
- CN      Cochlear Nucleus
- CNIC    Central Nucleus Inferior Colliculus
- COCB    Crossed Olivocochlear Bundle
- DAB     Diaminobenzidine
- DCIC    Dorsal Cortex Inferior Colliculus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN</td>
<td>Dorsal Cochlear Nucleus</td>
</tr>
<tr>
<td>DMPO</td>
<td>Dorsomedial Periolivary</td>
</tr>
<tr>
<td>DNLL</td>
<td>Dorsal Nucleus of the Lateral Lemniscus</td>
</tr>
<tr>
<td>DPOAE</td>
<td>Distortion Product Otoacoustic Emission</td>
</tr>
<tr>
<td>ECIC</td>
<td>External Cortex Inferior Colliculus</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>EP</td>
<td>Endocochlear Potential</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory Postsynaptic Potential</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic Acid Decarboxylase</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth-Associated Protein 43</td>
</tr>
<tr>
<td>GlyT2</td>
<td>Glycine Transporter 2</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>IC</td>
<td>Inferior Colliculus</td>
</tr>
<tr>
<td>IHC</td>
<td>Inner Hair Cell</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>INLL</td>
<td>Intermediate Nucleus of the Lateral Lemniscus</td>
</tr>
<tr>
<td>I/O</td>
<td>Input/Output</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory Postsynaptic Potential</td>
</tr>
<tr>
<td>LL</td>
<td>Lateral Lemniscus</td>
</tr>
<tr>
<td>LNTB</td>
<td>Lateral Nucleus of the Trapezoid Body</td>
</tr>
<tr>
<td>LOC</td>
<td>Lateral Olivocochlear</td>
</tr>
<tr>
<td>LSO</td>
<td>Lateral Superior Olive</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MGB</td>
<td>Medial Geniculate Body</td>
</tr>
<tr>
<td>MNTB</td>
<td>Medial Nucleus of the Trapezoid Body</td>
</tr>
<tr>
<td>MOC</td>
<td>Medial Olivocochlear</td>
</tr>
<tr>
<td>MSO</td>
<td>Medial Superior Olive</td>
</tr>
<tr>
<td>OAE</td>
<td>Otoacoustic Emission</td>
</tr>
<tr>
<td>OC</td>
<td>Olivocochlear</td>
</tr>
<tr>
<td>OCB</td>
<td>Olivocochlear Bundle</td>
</tr>
<tr>
<td>OHC</td>
<td>Outer Hair Cell</td>
</tr>
<tr>
<td>PTS</td>
<td>Permanent Threshold Shift</td>
</tr>
<tr>
<td>PVCN</td>
<td>Posterior Ventral Cochlear Nucleus</td>
</tr>
<tr>
<td>RPO</td>
<td>Rostral Periolivary</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SOC</td>
<td>Superior Olivary Complex</td>
</tr>
<tr>
<td>SPN</td>
<td>Superior Paraolivary Nucleus</td>
</tr>
<tr>
<td>TEOAE</td>
<td>Transient Evoked Otoacoustic Emission</td>
</tr>
<tr>
<td>TTS</td>
<td>Temporary Threshold Shift</td>
</tr>
<tr>
<td>UOCB</td>
<td>Uncrossed Olivocochlear Bundle</td>
</tr>
<tr>
<td>VCN</td>
<td>Ventral Cochlear Nucleus</td>
</tr>
<tr>
<td>VNLL</td>
<td>Ventral Nucleus of the Lateral Lemniscus</td>
</tr>
<tr>
<td>VNTB</td>
<td>Ventral Nucleus of the Trapezoid Body</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review and General Introduction

1.1. General Overview

The brain is the most complicated organ in mammals and consists of a series of complex interconnected networks, each with their own set of functions. The auditory system is one of these integrated networks in the brain and is composed of afferent (ascending) and efferent (descending) auditory pathways. The latter play an important role enabling the brain to modify afferent auditory input before it reaches the cortex. An important component of the efferent pathways is the olivocochlear (OC) system which originates in the brainstem and projects out to the organ of Corti within the cochlea. A subpopulation of the OC system, the MOC neurons, is thought to play a role in the discrimination and detection of signals in noise, protection against acoustic trauma and attention-focused sound detection (Scharf et al., 1994, Scharf et al., 1997, Rajan, 2000, Guinan, 2006, 2011). Neuroanatomical studies have established that MOC neurons en route to the cochlea give off collateral branches to the CN (Brown et al., 1988, Winter et al., 1989, Brown et al., 1991, Brown and Benson, 1992, Benson et al., 1996, Brown and Vetter, 2009). However, the targets of these collateral branches in the CN are as yet unknown since a conflict exists between results obtained from in vivo electrophysiological studies (Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009) and in vitro pharmacological studies (Fujino and Oertel, 2001).

As this study aims to identify which neural cell types in the CN receive synaptic innervation from the collateral branches of the MOC neurons, the anatomical structure and cellular distribution in each subdivision of the CN will be elucidated in the first section of this introduction. The second section focuses on the physiological properties and functional roles of the OC system in auditory processing. The origin, distribution, morphology and projections of the OC system in several species will be discussed in the third section. The last section provides a general overview of the potential roles of MOC collateral branches in hearing.
1.2. Anatomy of the Cochlear Nucleus

The CN receives and processes signals from auditory nerve fibres and transmits these signals to other nuclei throughout the brainstem and midbrain (Doucet and Ryugo, 1997). The CN is divided into the dorsal cochlear nucleus (DCN) and the ventral cochlear nucleus (VCN) which on the basis of the cellular morphology can be further separated into the posterior ventral cochlear nucleus (PVCN) and anterior ventral cochlear nucleus (AVCN) (Cajal, 1909, Lorente de Nó, 1933, Rose et al., 1959, Powell and Erulkar, 1962). The DCN is a laminated structure consisting of three layers in rats and four layers in mice and guinea pigs. These DCN layers and each of the subdivisions of the VCN contain a range of neuronal types of varied distribution (Harrison and Warr, 1962, Webster and Trune, 1982, Hackney et al., 1990).

The CN forms a prominent bulge on the dorsolateral side of the brainstem. The DCN is located on the dorsal aspect of the restiform body in contact with the floor of the lateral recess of the fourth ventricle. The VCN is located on the ventral aspect of the restiform body in the interval between the cochlear and vestibular fibres of the vestibulocochlear nerve (Hackney et al., 1990). The division between the PVCN and the AVCN is marked by the cochlear nerve root. The vestibular nerve is situated rostral to the cochlear nerve and extends into the brainstem at the level of the ventral portion of the AVCN (Hackney et al., 1990) (Figures 1.1 A-C).
Figure 1-1: Anatomical location of the cochlear nucleus (CN) and adjacent structures. A) Sagittal view of the guinea pig brainstem after removal of the cerebellum. (B) Guinea pig and (C) rat brainstem. Abbreviations: AVCN, anterior ventral cochlear nucleus; BIC, brachium of the inferior colliculus; Co, cochlea; CoN, cochlear nerve; Cp, cerebellar peduncle; Cx cerebral cortex; D, dorsal; DCN, dorsal cochlear nucleus; DC, dorsal cortex of the inferior colliculus; EC, external cortex of the inferior colliculus; IC, inferior colliculus; LL, lateral lemniscus; MGB, medial geniculate body; N5, trigeminal nerve; P, pons; PVCN, posterior ventral cochlear nucleus; Re, recess of the inferior colliculus; R, rostral; SC, superior colliculus; TB, trapezoid body; TrigN, trigeminal nuclei; VeN, vestibular nerve; IV, fourth ventricle (Hackney et al., 1990, Faye-Lund and Osen, 1985).

1.2.1. Fibre Tracts of the Cochlear Nucleus

The cochlear nerve fibres bifurcate after entering the VCN into ascending and descending branches (Figure 1.2). Fibres arising from the apical low frequency regions of the cochlea bifurcate primarily within the ventral regions of the VCN, whereas those arising from the basal high frequency regions bifurcate mainly within the dorsal regions of the VCN (Rose et al., 1960, Feldman and Harrison, 1969, Noda and Pirsig, 1974, Bourk, 1976, Bourk et al., 1981, Hackney et al., 1990). The ascending branches course anteriorly to the medial region of the AVCN and terminate in the globular cell area, the small spherical cell area and the large spherical cell area. The descending branches course posteriorly to the globular cell
area, multipolar cell area and octopus cell area of the PVCN and then run dorsally to the DCN (Noda and Pirsig, 1974, Hackney et al., 1990). Once they enter the DCN, fibres emanating from the apical low frequency regions of the cochlea turn to travel to the ventrolateral regions of the DCN, whereas those emanating from the basal high frequency regions proceed to the dorsomedial regions of the DCN (Hackney et al., 1990).

There are three efferent pathways from the CN known as the dorsal, intermediate and ventral acoustic striae. Axons of the dorsal acoustic stria, or stria of von Monakow, extend from the pyramidal and giant cells of the DCN and project primarily to the contralateral IC (Fernandez and Karapas, 1967, Adams and Warr, 1976, Adams, 1979). Axons of the intermediate acoustic stria, or the stria of Held, extend from the octopus and type II multipolar cells of the VCN and project predominantly to the contralateral ventral nucleus of the lateral lemniscus (VNLL) and CN, respectively (Harrison and Irving, 1966a, Smith and Rhode, 1989, Huffman and Covey, 1995). The axons of octopus cells in the intermediate acoustic stria also innervate the superior paraolivary nucleus (SPN) (Schofield, 1995). Axons of the ventral acoustic stria, or trapezoid body, extend from the bushy and type I multipolar cells of the VCN and project bilaterally to the SOC nuclei before innervating the contralateral dorsal nucleus of the lateral lemniscus (DNLL), VNLL and IC (Beyerl, 1978, Smith and Rhode, 1989, Smith et al., 1993a).

1.2.2. Cytoarchitectural Regions of the Cochlear Nucleus

Cajal first described morphologically distinct neuronal types in the CN in 1909. Lorente de Nó (1933) then described an additional 40 to 50 types in the CN of the cat. Since then, other investigators have used stains that have allowed visualisation of the perikarya and projections patterns of a number of neuronal types in the VCN and DCN of the cat (Osen, 1969, Brawer et al., 1974), rat (Harrison and Irving, 1965, 1966b), guinea pig (Pirsig, 1968, Wexler and Gulley, 1978, Moore, 1986) and mouse (Harrison and Irving, 1966a, Webster and Trune, 1982).

Based on cytoarchitectural features and projection patterns, the DCN can be divided into four layers in the guinea pig and mouse and three layers in the rat (Harrison and Warr, 1962, Webster and Trune, 1982, Hackney et al., 1990). In the rat, the AVCN can be divided into
three major regions I, II and III. The PVCN can also be divided into two regions IV, V and the posterior part of II (Harrison and Irving, 1966a, 1966b). Previous studies of the rat, guinea pig and mouse have shown that the VCN contains a variety of anatomically distinct neuronal classes including octopus, globular bushy and spherical bushy cells, while the DCN contains stellate, pyramidal (fusiform), cartwheel, vertical and elongated cells. Other types of cells, however, have also been found in both the VCN and DCN including granular, giant, small and multipolar cells (Figure 1.2) (Harrison and Irving, 1965, 1966a, 1966b, Webster and Trune, 1982, Hackney et al., 1990).

Figure 1-2: Distribution of cell types and fibre tracts in all subdivisions of the cochlear nucleus. Abbreviations: AVCN, anterior ventral cochlear nucleus; cap, small cell cap; CoN, cochlear nerve; C, caudal; DCN, dorsal cochlear nucleus; DFT, descending fibre tract; D, dorsal; lam, the granule cell lamina; oca, octopus cell area; PVCN, posterior ventral cochlear nucleus; sgri, superficial granule cell layer; TB, trapezoid body; VTT, ventrotubercular tract; VeN, vestibular nerve (Hackney et al., 1990).
1.2.2.1. Cells Types Restricted to the Ventral Cochlear Nucleus

Octopus cells are also known as k cells in the rat (Harrison and Irving, 1966b). Osen (1969) renamed these cells as octopus cells in the cat. Webster and Trune (1982) and Hackney et al. (1990) used the same term to describe these cells in guinea pigs and mice (Webster and Trune, 1982, Hackney et al., 1990). These cells form a horn-shaped region that is situated caudally in the dorsal regions of the PVCN. Nissl staining shows ovoid or D-shaped cell bodies that are about 25µm in diameter with a large pale nucleus and cytoplasm that contains finely dispersed Nissl substance. Long and thick primary dendrites emanate from one side of the octopus cell bodies. In Golgi staining, these dendrites can be seen to extend perpendicularly to the fibres of the descending root of the cochlear nerve (Osen, 1969, Webster and Trune, 1982, Hackney et al., 1990, Golding et al., 1995). The large axons of octopus cells leave the PVCN via the intermediate acoustic stria and project to the caudal part of the contralateral VNLL, the contralateral and ipsilateral dorsal and lateral periolivary nuclei and the dorsomedial periolivary (DMPO) nucleus on both sides of the brainstem (Harrison and Irving, 1966b, Osen, 1969, Adams and Warr, 1976, Friauf and Ostwald, 1988, Smith et al., 1993b, Schofield, 1995, Thompson, 1998).


Globular bushy cells are also known as g cells in the rat (Harrison and Warr, 1962, Harrison and Irving, 1966b). These cells are situated in the posterior AVCN, in the region of the nerve root and throughout most of the PVCN (Harrison and Warr, 1962, Webster and Trune, 1982, Hackney et al., 1990). In Nissl staining, globular bushy cells have elongated, oval shaped cell bodies with a nucleus that is placed eccentrically and cytoplasm that contains finely dispersed Nissl substance. In Golgi staining, globular bushy cells usually have one thick dendrite that branches profusely to form a complex tufted dendritic arborisation (Webster and Trune, 1982, Hackney et al., 1990). Globular bushy cells give rise to large axons that leave the VCN via the trapezoid body (Harrison and Warr, 1962, Tolbert et al., 1982, Smith
and Rhode, 1987) and terminate in the contralateral medial nucleus of the trapezoid body (MNTB) (Harrison and Warr, 1962, Harrison and Irving, 1965, Friauf and Ostwald, 1988). The terminal part of these axons in the MNTB is called the calyx of Held. The axons of globular bushy cells also project to the ipsilateral lateral periolivary nucleus (Tolbert et al., 1982, Kuwabara et al., 1991, Henkel and Gabriele, 1999), the contralateral VNLL, the contralateral DMPO nucleus, the contralateral SPN (Morest, 1968, Kuwabara et al., 1991), the medullary reticular formation (Warr, 1972, Kamiya et al., 1988) and the contralateral lateral and ventral periolivary nuclei (Cant and Benson, 2003). The axonal terminals that emanate from globular bushy cells contain large round synaptic vesicles (Smith et al., 1991, Spirou et al., 1998).

Spherical bushy cells are known as c cells in the rat (Harrison and Irving, 1965). Osen (1969) renamed these cells as spherical bushy cells. Densely packed close to the dorsal edge of the AVCN, the density of these cells reduces towards the acoustic nerve root. The cells range from 15-23μm in diameter and are intermingled with small and multipolar cells. The cytoplasm of spherical bushy cells contains Nissl granules that appear as a necklace surrounding the nucleus which located in the centre of the cell (Harrison and Irving, 1965, Webster and Trune, 1982, Hackney and Pick, 1986, Hackney et al., 1990). Golgi staining shows the cell body to be round or ovoid with one or two short thick primary dendrites that split into dense branches of varying orientation (Hackney et al., 1990). The axons of these cells leave the CN via the trapezoid body (Warr, 1966, Noort, 1969, Brownell, 1975) and project to the ipsilateral lateral superior olive (LSO) nucleus, the lateral part of the ipsilateral medial superior olive (MSO) nucleus, the medial part of the contralateral MSO nucleus and the nuclei of the lateral lemniscus (LL) (Warr, 1982, Shneiderman and Henkel, 1985, Cant and Casseday, 1986, Beckius et al., 1999, Cant and Benson, 2003). The synaptic terminals in the SOC contain round vesicles (Goldberg and Brown, 1969, Lindsey, 1975, Cant, 1984, Smith and Rhode, 1989). These axons also terminate in the ipsilateral and contralateral periolivary nuclei and in the contralateral ventral periolivary nuclei (Smith et al., 1993b).

1.2.2.2. Cells Types Restricted to the Dorsal Cochlear Nucleus

Molecular layer stellate cells are located in layer 1 and occasionally in layer 2 of the DCN. These cells have small cell bodies that appear round or ovoid shaped. The cells are
approximately 8-10µm in diameter with large pale nuclei. Nissl staining shows a thin rim of cytoplasm. Golgi staining shows thin primary dendrites, about 0.5µm in diameter, arising from the cell body (Harrison and Warr, 1962, Webster and Trune, 1982, Hackney et al., 1990).

Pyramidal (fusiform) cells are situated in layer 2 of the DCN. These cells have large bipolar cell bodies and Nissl staining shows a large nucleus with a lobulated nucleolus. The nucleus is often in the centre of the cell and the cytoplasm appears pale with clumps of Nissl substance. Golgi staining shows that these cells have two dendritic fields. The apical dendrites are spiny and project into layer 1 of the DCN while the basal dendrites are smooth and project into layer 3 of the DCN. The apical dendrites branch more frequently and are not smooth in appearance like the basal dendrites (Webster and Trune, 1982, Hackney et al., 1990). The axons of pyramidal cells leave the DCN via the dorsal acoustic stria (Fernandez and Karapas, 1967, Osen, 1972, Adams and Warr, 1976) and project to the ipsilateral and contralateral IC (Fernandez and Karapas, 1967, Adams, 1979, Brunso-Bechtold et al., 1981, Nordeen et al., 1983, Oliver, 1984) and the contralateral medial geniculate body (MGB) (Malmierca et al., 2002). In the IC, the axonal terminals contain small, round synaptic vesicles (Oliver, 1984, 1985).

Cartwheel cells are located in the boundary between layer 1 and 2 of the DCN. These cells have small round somata measuring approximately 15µm in diameter. Nissl staining shows the perikarya contain round, large and centrally placed nuclei with two or more nucleoli. The cytoplasm surrounding the nucleus contains fine and dispersed Nissl substance. The thick primary dendrites that arise from the bodies of the cartwheel cells are spiny and extend towards layer 1 of the DCN. Golgi staining shows several thick non–tapering primary dendrites that arise from the cell bodies and project towards the external ependymal-covered surface of the DCN (Webster and Trune, 1982, Hackney et al., 1990). Cartwheel cells are also known to send projections to the fusiform cells of the DCN (Berrebi and Mugnaini, 1991).

Vertical cells are located within layer 3 of the DCN. These cells have elongate or ovoid cell bodies (Hackney et al., 1990) measuring approximately 10µm across and 20µm long in Golgi staining. The dendrites of vertical cells spread horizontally along iso-frequency laminae
Lacy elongated cells are only present in the DCN and are intermingled amongst the fibres of the dorsal and intermediate acoustic striae. These cells have small and medium bipolar cell bodies. The soma has a small centrally placed nucleus and gives rise to one or two dendrites. The axons of lacy elongated cells project to the contralateral DCN and VCN of the brainstem (Webster and Trune, 1982, Hackney et al., 1990, Shore et al., 1992).

1.2.2.3. Cells Types Found in both the Dorsal and Ventral Cochlear Nucleus

Granule cells appear small and ovoid-shaped, measuring approximately 9µm in diameter. Nissl staining shows that these cells contain a relatively large round and pale nucleus with a centrally placed nucleolus and a very thin, almost invisible, rim of cytoplasm. The superficial granule layer covers most of surface of the VCN, with the exception of the cochlear nerve root. The granule cell lamina which separates the DCN from the PVCN is located at the junction of the superficial granule layer and layer 2 of the DCN. Golgi staining shows that granule cells have ovoid cell bodies and 1-4 dendrites that end in claw-like protuberances. Granule cells give rise to thin axons that run parallel to the surface of the DCN across layer 1 and 2 of the DCN (Webster and Trune, 1982, Hackney et al., 1990).

Giant cells, h cells, are also present in the VCN and DCN. These cells have large cell bodies, measuring approximately 35µm in diameter (Harrison and Irving, 1965, 1966b, Webster and Trune, 1982, Hackney et al., 1990). In the VCN, the giant cell somata appear multiangular-shaped with a nucleus that is centrally placed. The cytoplasm of these cells contains a very coarse Nissl substance (Hackney et al., 1990). Several long thin dendrites arise from the cell bodies (Harrison and Irving, 1966b). Giant cells in the DCN are situated in layer 3 and 4. The axons of giant cells project out of the DCN via the dorsal acoustic stria towards the IC on both sides of the brainstem (Adams and Warr, 1976, Adams, 1979, Oliver, 1984, Alibardi, 2000) and to the contralateral CN (Cant and Gaston, 1982, Schofield and Cant, 1996a).

Small cells in the DCN and VCN were first described in the rat as j cells (Harrison and Irving, 1966b), then renamed small cells by Osen (1969). Small cell bodies range from 12-15µm in
diameter and are found in all parts of the CN (Harrison and Irving, 1966b, Webster and Trune, 1982, Hackney and Pick, 1986, Hackney et al., 1990). One or two thin dendrites arise from the cell bodies, but no axons have been observed (Harrison and Irving, 1966b, Shore et al., 1992). These cells are scattered amongst the globular and spherical bushy areas of the VCN (Hackney et al., 1990) and a high proportion are found above the bifurcation of the cochlear nerve root region (Hackney and Pick, 1986). These cells are also found in the area located underneath the superficial granule cell layer, the granule cell lamina, superficial granule cell layer and in layers 1, 2 and 3 of the DCN (Hackney et al., 1990).

Multipolar cells are also located in both ventral and dorsal subdivisions of the CN. These cells are not a homogenous population. Multipolar cells were first described in the rat as d-cells, f-cells or l-cells (Harrison and Irving, 1965, 1966b). In the cat, guinea pig and mouse they have been named multipolar cells (Osen, 1969, Webster and Trune, 1982, Hackney et al., 1990). Multipolar cells are large and appear to form a heterogeneous population of cells in the VCN (Osen, 1969, Hackney et al., 1990). These cells have an angular cell body approximately 15-30µm in diameter and Nissl staining shows a centrally placed nucleus and cytoplasm that contains coarse or fine Nissl granules (Webster and Trune, 1982, Hackney et al., 1990). Golgi staining shows that the soma gives rise to several dendrites that leave the cell body at many different angles (Brawer et al., 1974, Webster and Trune, 1982, Hackney et al., 1990). On the basis of Golgi preparations, multipolar cells are also named stellate cells due to their appearance (Brawer et al., 1974, Webster and Trune, 1982). Two distinct populations of stellate cells have been described in the VCN. The first population is known as T-stellate cells, whereas D-stellate cells constitute the second population (Oertel et al., 1990).

T-stellate cells in mice are so named due to their projection through the trapezoid body (Oertel et al., 1990). These cells correspond to type I stellate cells and planar cells in cats and rats, respectively (Cant, 1981, Doucet and Ryugo, 1997, Gómez-Álvarez and Saldaña, 2016). T-stellate cells in the VCN are situated between the nerve root and the octopus cell area, with a few scattered anterior to the nerve root (Osen, 1969, Brawer et al., 1974, Oertel et al., 1990, Doucet and Ryugo, 1997, Gómez-Álvarez and Saldaña, 2016). The dendrites of T-stellate cells in mice often extend in parallel to the course of cochlear nerve fibres and end in highly branched tufts, indicative that T-stellate cells receive input from a comparatively
limited group of cochlear nerve fibres. The tips of T-stellate dendrites in mice consistently lie in the vicinity of granule cells (Oertel et al., 1990). T-stellate cells have local axonal collaterals in the PVCN and also in the fusiform cell layer of the DCN (Smith and Rhode, 1989, Oertel et al., 1990, Doucet and Ryugo, 1997, Ferragamo et al., 1998). The axons of T-stellate cells leave the CN via the trapezoid body and are thought to project to the ipsilateral and contralateral IC (Osen, 1972, Beyerl, 1978, Roth et al., 1978, Adams, 1979, Cant, 1982, Coleman and Clerici, 1987, Oliver, 1987, Oertel et al., 1990, Schofield and Cant, 1996b), the ipsilateral LSO (Doucet and Ryugo, 2003, Gómez-Álvarez and Saldaña, 2016) and the contralateral ventral nucleus of the trapezoid body (VNTB) (Darrow et al., 2012).

In mice, the dendritic orientation of T-stellate cells with respect to the tonotopy of the VCN (Rose et al., 1959, Rose et al., 1960, Evans and Nelson, 1973, Goldberg and Brownell, 1973, Kiang et al., 1975, Bourk et al., 1981) indicates that T-stellate cells are likely to be narrowly tuned (Oertel et al., 1990). In cats, T-stellate cells show chopper responses to tone bursts with a steady firing pattern (Smith and Rhode, 1989). These responses can be further subdivided into sustained or transient chopper responses. Chopper cells in cats are sparsely innervated on the cell body (Smith and Rhode, 1989). The synaptic endings of chopper cells have round vesicle, indicative of excitatory synapses (Oliver, 1987, Smith and Rhode, 1989, Palmer et al., 1996). The response properties of chopper cells suggest that they might be implicated in encoding complex auditory stimuli, necessary for the discrimination of speech sounds (Rhode and Smith, 1986, Blackburn and Sachs, 1989, May et al., 1998, Oertel et al., 2011).

One study shows that D-stellate cells show onset chopper response properties when categorized according their responses to sound (Smith and Rhode, 1989). D-stellate cells in mice are so named for the dorsalward projection of their axons (Oertel et al., 1990). However, the exact trajectory of the axon of D-stellate cells is still not established. In cats, Smith and Rhode (1989) showed that the axon of onset chopper (D-stellate) cells leaves the CN via the intermediate acoustic stria. Other researchers, in cats, guinea pig and mice, suggested that the onset chopper (D-stellate) cells exit the CN via the dorsal acoustic stria (Arnott et al., 2004, Smith et al., 2005, Brown et al., 2013). Indeed, data on the trajectories of the axons of onset chopper cell described in the previous studies are based on a very small sample of intracellulary injected cells with incomplete axonal filling. However, as
shown in this thesis, biocytin injections at the floor of the fourth ventricle do not label multipolar (D-stellate) cells in the PVCN and AVCN even in the case where labelled cells are found in the DCN (Chapter 3). Therefore, it seems likely that the axon of D-stellate cells runs deeper than the dorsal acoustic stria. D-stellate cells are probably equivalent type II stellate cells and large multipolar cells in cats and rats, respectively (Cant, 1981, Doucet and Ryugo, 1997). The dendrites of D-stellate cells in mice are long, smooth, tapered and sparsely branched and span across multiple iso-frequency laminae (Oertel et al., 1990). The tips of D-stellate dendrites are often located within the superficial granule cells or in the granule cell lamina which separates the DCN from the PVCN (Mugnaini et al., 1980, Oertel et al., 1990). Axonal collaterals of D-stellate cells are intermingled with T-stellate cells in the VCN and invaded the granule cell regions (Smith and Rhode, 1989, Oertel et al., 1990, Doucet and Ryugo, 1997, Ferragamo et al., 1998). D-stellate cells are believed to project to the contralateral DCN and PVCN and the ipsilateral DCN, on the basis of their morphological properties and the direction of their axonal projections (Adams and Warr, 1976, Cant and Gaston, 1982, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Oertel et al., 1990, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996b, Alibardi, 1998a, Palmer et al., 2003, Arnott et al., 2004, Doucet et al., 2009).

D-stellate cells would be broadly tuned because of orientation of the dendrites across the incoming array of auditory nerve fibres. The span of D-stellate dendrites in mice and the properties of corresponding cells in cats suggest that D-stellate cells are broadly tuned (Smith and Rhode, 1989, Oertel et al., 1990). D-stellate cells are thought to be glycinergic inhibitory cells (Wenthold et al., 1987, Smith and Rhode, 1989, Ferragamo et al., 1998, Doucet et al., 1999). The terminals of onset chopper cells in the ipsilateral and contralateral CN contain pleomorphic vesicles, suggestive of inhibitory synapses (Smith and Rhode, 1989). Onset chopper cells have been suggested to provide wideband inhibition to other cells in the VCN and DCN and to play a pivotal role in the detection of spectral cues related to sound identification or localization (Nelken and Young, 1994, Arnott et al., 2004) and detection of complex signals in noisy environments (Verhey et al., 2003, Neuert et al., 2004).
1.3. The Olivocochlear Efferent System

The auditory system is composed of afferent and efferent auditory pathways. The afferent pathways extend from the cochlea to the CN in the brainstem. The axons of the afferent cells project from the CN to the SOC, LL, IC, MGB and the auditory cortex (AC). The efferent pathways are organised in parallel to the afferent axons and extend from the AC to MGB, IC and LL. Efferent axons also emerge from the SOC nuclei and project down toward the organ of Corti within the cochlea (Held, 1926, Lorente de Nó, 1937, Rasmussen, 1946, 1953, Kimura and Wersäll, 1962, Morrison et al., 1975, Ginzberg and Morest, 1984, Spoendlin, 1985, Liberman et al., 1990).

Early anterograde degeneration studies found that efferent innervation of the organ of Corti is provided by the olivocochlear bundle (OCB) which originates from cells in the SOC of the mammalian brainstem (Rasmussen, 1946, 1953). Injections of the retrograde tracer, horseradish peroxidase, into one cochlea confirmed these findings (Warr, 1975). Warr and Guinan (1979) then used the retrograde axonal transport method to demonstrate that the efferent innervation of the organ of Corti consists of two separate populations that arise from cells in the SOC and project either to the OHCs or to the dendrites of auditory nerve fibres beneath the inner hair cells (IHCs). The first population of neurons is called the MOC neurons and they are mostly located in the medial regions of the SOC. The second population of neurons is called the lateral olivocochlear (LOC) neurons and they are located in the lateral regions of the SOC (Warr and Guinan, 1979).

1.3.1. Physiology of the Olivocochlear Efferent System

Animal experiments have demonstrated that electrical stimulation at the floor of the fourth ventricle activates the OCB. Understanding the mechanical and neural effects produced by stimulation in medial and lateral efferents of the OCB is crucial for interpreting the role of these efferents in hearing (Galambos, 1956, Fex, 1959, Desmedt, 1962, Wiederhold and Peake, 1966, Buño and García-Ausitt, 1970, Wiederhold, 1970, Teas et al., 1972, Gifford and Guinan, 1983, Mulders et al., 2002). Following electrical stimulation of the OCB at the floor of the fourth ventricle, the effects on the gross cochlear potential and changes in the firing patterns of single auditory nerve fibres are mainly because of the action of MOC efferents.
MOC axons are myelinated and thick which facilitates both recording and electrical stimulation of MOC fibres. In contrast, the thin unmyelinated axons of the LOC neurons are difficult to record from or to stimulate electrically (Gifford and Guinan, 1983, 1987, Guinan, 1996).

1.3.1.1. Effects of the Medial Olivocochlear System


MOC neurons give rise to axons that synapse on OHCs. The primary neurotransmitter released by MOC presynaptic terminal is acetylcholine (ACh) (Guinan et al., 1983). Two subunits of the nicotinic ACh receptor family, alpha-9 and alpha-10, are expressed by the OHCs (Elgoyhen et al., 1994, Glowatzki et al., 1995, Elgoyhen et al., 2001, Morley and Simmons, 2002). Stimulation of MOC efferents leads to activation of the nicotinic ACh receptors (Figure 1.3). This activation allows calcium ions to flow into the OHCs through ligand-mediated calcium channels (Fuchs and Evans, 1990, Fuchs et al., 1990). Persistent entry of calcium ions into the OHCs leads to activation of calcium-activated potassium channels (SK channels) which hyperpolarize the OHCs via the outflow of potassium ions (Fuchs and Evans, 1990, Housley and Ashmore, 1991, Evans, 1996, Blanchet et al., 2000, Lustig, 2006). Hyperpolarization of the OCHs leads to inhibition of the active feedback process of the OHCs and results in a reduction of the vibration of the basilar membrane in response to sound (Patuzzi and Rajan, 1990, Murugasu and Russell, 1996, Dallos et al., 1997, 2000).
Dolan et al., 1997, Russell and Murugasu, 1997, Brownell et al., 2001, Cooper and Guinan, 2006). This reduction leads to a decreased neurotransmitter release from the IHCs to afferent dendrites and thus reducing the size of the CAP amplitude of the auditory nerve fibres (Guinan, 1996). Release of ACh from the MOC terminals increases the basolateral wall conductance of the OHCs. This increase in OHC synaptic conductance shunts the current in the hair cells and therefore increases the magnitude of the alternating current flow via the OHCs, which leads to an increase in the amplitude of CM (Art et al., 1984, Fuchs and Evans, 1990, Housley and Ashmore, 1991, Guinan, 1996, Russell and Murugasu, 1997).

The EP which is normally between +80-100mV in the scala media of the inner ear falls by up to 3mV after MOC activation (Fex, 1959, Gifford and Guinan, 1987). The drop in the standing voltage above the OHCs contributes to the reduction in cochlear amplification (Guinan and Stankovic, 1996). The reduction in the EP also leads to a reduction in the standing current that flows from the endolymphatic space of the inner ear through stereocilia of the IHCs (Guinan, 2011). As a result, the IHCs will hyperpolarize and the amount of spontaneous neurotransmitter release to afferent fibres will reduce and this leads to suppression of spontaneous activity of the primary afferents.

Apart from electrical stimulation, there is ample evidence to indicate that MOC neurons can be activated by acoustic stimulation of the ipsilateral and/or contralateral ear (Buño, 1978, Robertson and Gummer, 1985, Liberman and Brown, 1986, Folsom and Owsley, 1987, Liberman, 1989). The combination of intracellular injection of horseradish peroxidase and
recording of single efferent activity has been used to study the correlation between the anatomy and physiology of MOC neurons (Robertson and Gummer, 1985, Liberman and Brown, 1986). Recordings from single MOC fibres indicated that MOC fibres have low, regular discharge rates and long latency. MOC fibres, moreover, have sharp tuning curves that are very similar to auditory nerve fibres. Data from single MOC fibres labelled by dye injection indicate that each MOC fibre innervates a cochlear frequency region near to the MOC fibre’s characteristic frequency (Robertson and Gummer, 1985, Liberman and Brown, 1986).

Sound-evoked MOC activity is achieved via the MOC acoustic reflex pathway. Acoustic stimulation to the cochlea causes excitation of the primary afferent neurons. These afferents innervate reflex interneurons in the ipsilateral PVCN. The axons of the PVCN cells cross the midline to innervate the contralateral MOC neurons. These MOC neurons then send crossed or uncrossed axons back to the contralateral and ipsilateral cochleae, respectively. The MOC responses recorded in the contralateral cochlea after presentation of sound to the ipsilateral ear are mediated by the crossed MOC axons. Those responses recorded in the ipsilateral cochlea after presentation of sound to the contralateral ear are mediated by the uncrossed MOC axons (Brown et al., 2003, Guinan, 2006). Like the results obtained from studies using electrical activation of MOC fibres, sound delivered to the contralateral ear leads to suppression of the CAP of the auditory nerve as well as inhibition of the activity of auditory primary afferent fibres (Buño, 1978, Liberman, 1989, Warren and Liberman, 1989, Puria et al., 1996).

1.3.1.2. Effects of the Lateral Olivocochlear System

The unmyelinated and thin axons of LOC neurons are difficult to stimulate experimentally by electrically stimulating at the floor of the fourth ventricle (Ranck, 1981, Gifford and Guinan, 1987) and their unmyelinated and thin axons make it difficult to record their acoustic responses (Robertson and Gummer, 1985). As a result, the peripheral effects of the LOC system are very poorly understood. Many studies have used de-efferentation methods in an attempt to improve understanding of the effects of the LOC system on cochlear output. The LOC efferents in guinea pig project predominately (99%) to the ipsilateral cochlea, while the remaining 1% of these neurons project contralaterally (Robertson, 1985). Hence, long-
lasting changes in cochlear neural responses are usually observed in the cochlea ipsilateral to the LOC lesions.

After the OCB in cats was sectioned at the floor of the fourth ventricle, Liberman (1990) noted that the spontaneous discharge rates of auditory nerve fibres were decreased. Likewise, after chronic and complete sectioning of the OCB at the level of the internal auditory meatus in adult chinchillas, Zheng et al. (1999) reported a decrease in the spontaneous activity of auditory afferent fibres. The decrease in the spontaneous discharge rates of auditory nerve fibres in both studies could conceivably be due to the loss of LOC tonic activity. These results suggest that LOC efferents may have an excitatory action on the primary afferent dendrites beneath the IHCs.

Indirect activation of LOC efferents has been recently achieved by electrical stimulation in the IC of anaesthetised guinea pigs (Groff and Liberman, 2003). Based on the site of the IC stimulation, cochlear neural responses could be either enhanced or suppressed by effects which were attributed to the actions of the lateral system without effects on cochlear responses dominated by the OHCs. To support this notion, Groff and Liberman (2003) have shown novel changes in cochlear neural responses which are basically different from those observed when stimulating the OCB at the floor of the fourth ventricle. The spontaneous rate of the auditory afferents nerve was enhanced (Darrow et al., 2006) or suppressed (Liberman, 1990, Le Prell et al., 2003) after LOC neurons were lesioned.

After electrical stimulation of the IC in guinea pigs, Mulders and colleagues noted an excitatory effect on cochlear potentials that might be caused by the actions of LOC system (Mulders and Robertson, 2005). Based on the study of Mulders and colleague (2005), it is possible that the activation of LOC efferents by using electrical stimulation of IC results in both excitatory and inhibitory effects in cochlear neural responses. Based on anatomical evidence, the two LOC groups, intrinsic and shell neurons, contain different neurotransmitters which may produce different effects on cochlear neural response (Groff and Liberman, 2003, Mulders and Robertson, 2005).

Dopamine is one of the major neurotransmitters of the lateral efferent system (Fex et al., 1986, Jones et al., 1987, Eybalin et al., 1993, Gil-Loyzaga et al., 1997, Ruel et al., 2001,
Maison et al., 2003). A number of previous studies have identified a wide range of dopamine receptor subtypes in the cochlea, including D₁/D₅, D₂, and D₃ receptors (Karadaghy et al., 1997, Inoue et al., 2006, Niu and Canlon, 2006). After perilymphatic perfusion with D₁/D₅, D₂, and D₃ receptor agonists and antagonists, Garrett et al. (2009) studied the effects of the receptors on the cochlea of guinea pig. This study has shown suppression in the CAP amplitudes following perilymphatic perfusion with D₁/D₅ agonists and D₂ antagonists. They also found perfusion with D₂ antagonist caused a marked and persistent reduction in the summing potential (CM and distortion product otoacoustic emissions (DPOAE) amplitudes) suggesting that the effect of the D₂ receptor is probably limited to the hair cells and not to the primary afferent dendrites (Garrett et al., 2009). There was, however, no alteration in the amplitude of the CAP and summing potential following perfusion of the cochlea with D₃ receptor agonists and antagonists. These results suggest divergent effects of dopamine dependent on the receptor class activated (Garrett et al., 2009).

1.3.1.3. Functional Role of the Medial Olivocochlear System

1.3.1.3.1. Signal Detection in Noise

The MOC system is thought to play a role in the discrimination and detection of signals in noise. In a quiet background, the effect of MOC stimulation is to inhibit primary afferent responses by reducing the cochlear amplifier gain. In a noisy background, activation of MOC efferents enhances the responses of primary afferents to the tone (Dolan and Nuttall, 1988, Winslow and Sachs, 1988, Kawase and Liberman, 1993). Figure 1.4A shows the response of a primary afferent to tone bursts at characteristic frequency in a quiet background without MOC activation. The response of primary afferents increases with tone-burst sound levels until it reaches a saturation rate. In a quiet background with MOC activation, the input/output (I/O) curve of primary afferent response is shifted to the right, as shown in Figure 1.4B.

When background noise is present and MOC is not activated, the background firing rate of primary afferents is raised in the response to the continuous background noise (Figure 1.4C). This continuous increase in firing rate causes adaptation in the response of primary afferents, possibly because the neural transmitter supply of the IHCs is depleted. The result
is lowering of primary afferent firing rate to high tone-burst levels. Therefore, in the presence of background noise, the dynamic range of primary afferent responses to the tone is reduced.

The response of primary afferents to background noise is reduced with MOC activation (Figure 1.4D). This leads to an increase in the saturation rate of primary afferents at high tone-burst levels due to less adaptation, so higher firing rates can be evoked by high tone-burst levels. The slope of the initial rising phase of the I/O curve increases, because the neural transmitter supply of IHCs is restored and the dynamic range of primary afferent responses to short tone-burst levels is partially restored to normal. This phenomenon is called MOC unmasking, or anti-masking effect. This mechanism could theoretically improve the detection and discrimination of transient sounds in noisy environments (Winslow and Sachs, 1987, 1988, Kawase and Liberman, 1993, Guinan, 1996, 2006, 2011).

![Figure 1-4: Schematic representation of medial olivocochlear (MOC) stimulation on input-output functions of primary afferent responses to characteristic frequency tone bursts in quiet (A and B) and noisy backgrounds (C and D) (Guinan, 1996).](image)

The effect of anti-masking on the primary afferent response in the presence of background noise following MOC activation has been confirmed in many studies (Wiederhold and Kiang, 1970, Winslow and Sachs, 1987, Kawase and Liberman, 1993). Consistent with this notion,
anti-masking effects of MOC activation on tone responses in continuous background noise can also be observed in both the CN (Mulders et al., 2008) and IC (Seluakumaran et al., 2008b). In a substantial number of CN and IC cells, activation of the MOC system resulted in increased slope and dynamic range of neuronal I/O functions, consistent with an improvement in intensity discrimination of the pure tones in noise.

The role of MOC efferents in the discrimination and detection of signals in a noisy background has been tested either by cutting MOC efferent fibres or by looking for a relationship between the MOC activation and psychophysical performance (Guinan, 2006, 2011). Behavioural studies have been proposed that lesions of the MOC efferents in cats produced significant deficit in the discrimination of sound intensity when behavioral performance was tested in the presence of background noise (May et al., 1995). Dewson (1968), rather than using simple tones, trained rhesus monkeys to discriminate vowel sounds and found that after OCB lesions, the ability of those animals to discriminate vowel sounds in noise was significantly deteriorated. Likewise, Hienz and colleagues (1998) observed that lesions of OCB in cats produced deficit in discrimination of vowel sounds in noise, especially in the presence of high levels of background noise (Hienz et al., 1998). Both studies in rhesus monkeys (Dewson, 1968) and cats (May et al., 1995, Hienz et al., 1998) suggest that MOC system could play a crucial role in detection and discrimination of more complex sounds and not only simple tones in a noisy background.

In humans, vestibular neurectomy in patients in whom the OCB was severed showed the importance of MOC efferents in speech-in-noise intelligibility (Zeng and Shannon, 1994, Giraud et al., 1997, Zeng et al., 2000). Nevertheless, Scharf and colleagues did not notice any deterioration in the discrimination and detection of signals in a noisy background for those patients with the vestibular nerve cut, see below with regard to attentional effects of neurectomy (Scharf et al., 1994, Scharf et al., 1997). These conflicting results may be due to the fact that vestibular neurectomy disrupts MOC efferent axons to varying degrees or MOC efferents may not be cut totally in those patients (Scharf et al., 1994, Zeng and Shannon, 1994, Giraud et al., 1995, Scharf et al., 1997, Chays et al., 2003).

Psychophysical experiments in humans have been carried out to examine the role of the MOC efferent activation in the discrimination and detection of signals in a noisy
background. Activation of MOC-reflex by the addition of contralateral noise assisted in
MOC-reflex activation reduced the intensity differences limens of tones in noise in the
ipsilateral ear (Micheyl et al., 1997). Contralateral acoustic stimuli in children (Kumar and
Vanaja, 2004) and adults (Giraud et al., 1997) enhance speech-in-noise intelligibility for the
ipsilateral ear. Mounting evidence suggests that the MOC system plays an important role in
the discrimination and detection of signals in noise; however, more investigations are
required in both humans and animals.

1.3.1.3.2. Protection from Acoustic Trauma

A second proposed role for the MOC system is to protect the cochlea from damage during
intense acoustic exposure (Rajan, 2000). Excessive exposure to loud sound can cause
temporary threshold shifts (TTS) in primary afferent. Many studies have demonstrated that
electrical stimulation of the crossed olivocochlear bundle (COCB) at the floor of the fourth
ventricle can reduce the TTS (Rajan, 1988, Rajan and Johnstone, 1988). It has also been
shown that contralateral acoustic stimulation (Cody and Johnstone, 1982, Rajan and
Johnstone, 1988, Reiter and Liberman, 1995) and stimulation of the contralateral IC (Rajan,
1990) help to significantly reduce TTS. Patuzzi and Thompson (1991) observed that after
sectioning the MOC efferents at the floor of the fourth ventricle of guinea pigs, the
protective effect of contralateral sound (10 kHz, 80 dB SPL) during intense acoustic
overstimulation (10 kHz, 115 dB SPL for 1 minute) disappeared. They also found the
intersubject variability in TTS was reduced, suggesting that the MOC efferents have differing
tonic effects on normal cochlear function across animals (Patuzzi and Thompson, 1991).

Rajan and Johnstone (1988), in guinea pigs, demonstrated that the intensity of the
traumatising acoustic stimuli is proportional to the extent of the MOC protective effect.
Electrical stimulation of the OCB at the same time as acoustic overstimulation produces
significant reduction in the TTS just for short duration acoustic exposures of one and two
minutes (Reiter and Liberman, 1995). Based on these observations, Reiter and colleague
(1995) proposed that the protection by the MOC system in the cochlea is mediated by the
“slow” effects of MOC stimulation rather than the classic “fast” effects. This is because the
slow MOC effect can only be maintained for 1-2 minute of continuous MOC stimulation
whereas the fast MOC effect remains fundamentally undiminished during this time frame (Reiter and Liberman, 1995).

Midline lesions of the OCB in both chinchillas and guinea pigs showed that animals were more susceptible to permanent threshold shifts (PTS) from acoustic trauma compared to control animals (Liberman and Gao, 1995, Kujawa and Liberman, 1997, Zheng et al., 1997a, Zheng et al., 1997b). The MOC reflex strength can be measured through contralateral suppression of otoacoustic emissions (OAEs). Maison and Liberman (2000) measured OAE suppression in guinea pigs that were then exposed to traumatizing sounds and measured OAE. Guinea pigs were classified into those with weak, average and strong MOC reflex. The animals with the weakest MOC reflex had the largest PTS while the animals with the strongest MOC reflex had the smallest PTS, suggesting that activity in the MOC system reduces the PTS and protects the ear from permanent acoustic trauma (Maison and Liberman, 2000). Overexpression of alpha9 nicotinic ACh receptors in the termination of MOC efferents in the cochlea (i.e. OHCs) can considerably reduce the TTS and PTS resulting from acoustic injury (Maison et al., 2002). All the above evidence and observations suggest that the MOC system plays a crucial role in preventing acoustic trauma caused by intense sound.

1.3.1.3.3. Auditory Attention

Based on behavioural experiments, it has been suggested that the MOC efferents contribute to the modulation of cochlear responses during attentional processes. Oatman (1971) showed in cats that the CAP of the acoustic nerve was reduced during visual attention. Other experiments have shown significant reductions in the measurements of the cochlear responses at the round window during visual attention (Oatman, 1971, 1976, Glenn and Oatman, 1977, Oatman and Anderson, 1977). Brix (1984) studied the effect of attention on the auditory brainstem evoked responses (ABER) and found that selective attention to the auditory stimulation resulted in decrease in the interpeak latency times of wave I to wave V of the ABER.

Scharf and colleagues have proposed another role for the MOC system in attention processes (Scharf et al., 1994, Scharf et al., 1997). In patients who had surgical cuts of the
vestibular nerve, lacking the MOC efferents, Scharf and colleagues measured the auditory capacities for those patients before and after a vestibular neurectomy, including measurements of pure tone threshold, loudness adaptation, intensity discrimination and the difference in detection rate between expected and unexpected near-threshold tones observed in the presence of background noise. The only obvious change after a vestibular neurectomy was that almost all participants can better detect unexpected signals in noise after the surgery. The change that was observed in the capacity of those patients in the detection of unexpected signals in a background noise suggests an impaired ability to focus attention in the frequency domain. Scharf and colleagues, therefore, proposed that MOC efferents could play a role in improving selective attention in the auditory system (Scharf et al., 1994, Scharf et al., 1997).

The final evidence for the role of the MOC system in attention processes concerns the relationship between MOC system and the AC. The AC has been shown to be a source of descending auditory projections that directly contact the MOC neurons (Mulders and Robertson, 2000a). Khalfa et al. (2001) studied the effect of the absence of the AC in one hemisphere on the peripheral auditory system in patients with temporal lobe epilepsy. The epileptogenic region in such patients was located in the superior temporal gyrus which was determined by the use of stereo-encephalography. The AC constitutes a substantial part of the superior temporal gyrus. Khalfa and colleagues (2001) by means of transient evoked otoacoustic emissions (TEOAEs) examined the MOC system function before and after partial or total resection of the superior temporal gyrus, including the primary and secondary auditory cortical areas. This study found that the amplitude of TEOAEs was clearly reduced after partial or total removal of the superior temporal gyrus (Khalfa et al., 2001). Another study also found that after electrical stimulation of the contralateral AC in epileptic patients, the amplitude of TEOAEs was remarkably decreased (Perrot et al., 2006). These studies suggest that the AC plays a role in modulating auditory periphery activity via the MOC system. Therefore, it can be hypothesised that the MOC system might be able to mediate cortical tasks such as attention by influencing the OHCs responses (Khalfa et al., 2001, Perrot et al., 2006).
1.3.1.3.4. Perceptual Learning

One possible role for the MOC system in hearing is consistent with it being a part of perceptual learning as a result of auditory training. A series of studies have shown that auditory training plays an important role on MOC reflex strength. In one study, Perrot et al. (1999) compared MOC reflexes in professional musicians and subjects with no particular musical experience, and showed that subjects with no particular musical experience have weaker MOC reflexes than subjects who were professional musicians. In another study, MOC activity in normal-hearing adult listeners was measured during 5-day training regimen on monaural consonant-vowel phoneme-in-noise discrimination task (de Boer and Thornton, 2008). Over the training period, not only were there significant improvements in the speech-in-noise task but there were also obvious increases in MOC activity in listeners who had weaker MOC activation (de Boer and Thornton, 2008). Children with reading difficulties have also been studied by Veuillet et al. (2007). In this study, some children showed an absence of the asymmetry in favour of the right ear which was found in average-reading children. After audio-visual training, a significant improvement in reading scores was shown in these children and MOC functioning showed increased asymmetry in favour of the right ear and this asymmetry became closer to normal (Veuillet et al., 2007). This suggests that one function of the MOC system may be to aid in perceptual learning; however, more investigation is required to prove that MOC activity increases because of perceptual learning, not as a by-product of other central changes (Guinan, 2011).

1.3.1.3.5. Homeostatic Regulation

One of the other proposed roles for the MOC system is to provide cochlear homeostasis. Patuzzi (2002), suggested that the MOC system might be involved in regulation the EP in the cochlea. Neurotransmitter release from the hair cells in the cochlea as well as the neural firing rate might be altered in response to any fluctuation of the EP. An increase in neural spontaneous activity due to the changes of EP might lead to generate phantom auditory sensation, tinnitus (for more details, see Section 1.5.3. (Mulders, 2006)). To keep the EP constant and avoid the phenomenon of cochlear tinnitus, it has been proposed that MOC activation can lead to lower the EP when it is too high and maintain the homeostatic regulation of the cochlea (Brown and Nuttall, 1984, Guinan, 1996, Patuzzi, 2002). Another
role put forward for the MOC system is to control the cochlear gain. The effect of electrical stimulation of the MOC system on the mammalian cochlear amplifier permits the control of dynamic range of hearing. As mentioned earlier in Section 1.3.1.3.1., with MOC activation the I/O curve of primary afferents is shifted to the right, i.e. to the higher tone burst sound intensities, thus allowing representation of sounds at high tone burst sound levels (Geisler, 1974, Guinan, 1996).

1.3.1.4. Functional Role of the Lateral Olivocochlear system

The functional significance of the LOC system in hearing is not well investigated and is not the focus of our research so the role of the LOC system in hearing will only be discussed briefly. A series of studies conducted on the effects of de-efferentation of the LOC system found that the spontaneous discharge rates of the primary afferent fibres were decreased, which suggested an excitatory role for the LOC system (Liberman, 1990, Walsh et al., 1998, Zheng et al., 1999). The efferent feedback from the LOC system has been proposed to produce a change in the adaptation level of primary afferents and maintain the binaural balance of cochlear activation (Groff and Liberman, 2003). Such a mechanism would be important in the detection of interaural intensity differences for accurate sound localisation and this may also be an important role of the LOC system. After destruction of the LSO neurons, Darrow et al. (2006) found that the interaural correlation of the cochlear neural response amplitudes was removed. In another experiment, damaging the cell bodies of the LOC system in mice made the cochlea more susceptible to acute acoustic injury (Darrow et al., 2006). Based on this observation, it is possible to suggest that the LOC system may play a role in the protection of the cochlea from acoustic trauma (Ruel et al., 2001, Darrow et al., 2006).

1.4. Anatomy of the Olivocochlear Efferent System

Several studies have been conducted to determine the origin, distribution, morphology and projections of LOC and MOC neurons in the guinea pig (Robertson, 1985, Robertson et al., 1987a, Robertson et al., 1987c, Aschoff and Ostwald, 1988), cat (Warr, 1975, Warr and Guinan, 1979, Guinan et al., 1983, Arnesen and Osen, 1984, Guinan et al., 1984, Warr et al., 2002), rat (White and Warr, 1983, Aschoff and Ostwald, 1988, Vetter and Mugnaini, 1992),
hamster (Sánchez-González et al., 2003), mouse (Campbell and Henson, 1988), bat (Bishop, 1986, Aschoff and Ostwald, 1987, Bishop and Henson, 1987) and squirrel monkey (Thompson and Thompson, 1986).

The origin of LOC neurons varies across species. An early study in cats has shown that LOC neurons are mostly found in the dorsal hilus of LSO (Warr, 1975), although some are also found in the anterolateral and dorsolateral periolivary nuclei of the SOC (Warr et al., 2002). A later study in cats has shown that LOC neurons can be divided into two populations based on their proximity to, and dendritic association with, the LSO. The first population has intimate contact with the LSO and is called marginal-LOC neurons. The second population is further from the LSO and called para-LOC neurons (Warr et al., 2002). In squirrel monkeys and Rhinolophus rouxi bats, LOC neurons are situated between the LSO and MSO (Thompson and Thompson, 1986, Aschoff and Ostwald, 1987). In rodents and all bat species except Rhinolophus rouxi, the cell bodies of LOC neurons are found within the LSO (Robertson, 1985, Bishop, 1986, Aschoff and Ostwald, 1987, Bishop and Henson 1987, Robertson et al., 1987a, Robertson et al., 1987c, Aschoff and Ostwald, 1988, Campbell and Henson, 1988, Vetter and Mugnaini, 1992, Sánchez-González et al., 2003).

In rats, LOC neurons have been subdivided into two populations: intrinsic and shell neurons (Vetter and Mugnaini, 1992). Shell LOC neurons are large, multipolar and located at the margins of the LSO nucleus. Intrinsic LOC neurons are small and have two subpopulations: simple and converging intrinsic neurons (Warr et al., 1997). These neurons are fusiform and are found within the body of the LSO nucleus (Vetter and Mugnaini, 1992).

The exact location of MOC neurons in the SOC also varies across species. In cats, the cell bodies of MOC neurons originate from the lateral nucleus of the trapezoid body (LNTB), the DMPO nucleus and the VNTB (Warr, 1975, Warr and Guinan, 1979, Guinan et al., 1983). In rats, hamsters and mice, MOC neurons are located in the VNTB (White and Warr, 1983, Aschoff and Ostwald, 1988, Campbell and Henson, 1988, Vetter and Mugnaini, 1992, Sánchez-González et al., 2003). In guinea pigs, MOC neurons are found in the rostral periolivary (RPO) nucleus, the DMPO nucleus and the VNTB (Robertson, 1985, Robertson et al., 1987a, Robertson et al., 1987c). Some bat species, such as Rhinolophus rouxi, lack MOC neurons in their brainstem altogether (Aschoff and Ostwald, 1987).
In addition to variations in the locations of LOC and MOC neurons across species, the total number of efferent neurons that innervate the IHCs and OHCs of the organ of Corti also varies among species. Hamsters have approximately 341 OC neurons, the rat has about 976, cats have around 1366 and there are about 2345 OC neurons in the guinea pig (Warr, 1992, Sánchez-González et al., 2003). Cats and mice have approximately 65% LOC and 35% MOC neurons (Arnesen and Osen, 1984, Campbell and Henson, 1988, Warr et al., 2002). In humans, the split is 70% LOC and 30% MOC neurons (Arnesen, 1984). Intrinsic LOC neurons number about 85% of all LOC neurons in rats (Vetter and Mugnaini, 1992). In guinea pigs, the numbers of LOC and MOC neurons are roughly equal (Aschoff and Ostwald, 1987).

LOC and MOC neurons in the SOC have different sizes and shapes. LOC neurons are smaller than the MOC neurons. The cell morphology of LOC neurons is fusiform, with two opposite polar primary dendrites. MOC neurons are multipolar or stellate, with three or four primary dendrites (Aschoff and Ostwald, 1987, Cantos et al., 2000).

Both LOC and MOC neurons exhibit a positive reaction for choline acetyltransferase (ChAT). They also stain positive for acetylcholinesterase (AChE), but the intensity of staining of the MOC neurons is more marked than of LOC neurons. LOC neurons, their axons and their dendrites stain positive for glutamic acid decarboxylase (GAD), calcitonin gene-related peptide (CGRP) and tyrosine hydroxylase (Schuknecht and Nomura, 1965, Osen et al., 1984, Thompson and Thompson, 1986, Vetter et al., 1991, Darrow et al., 2006, Brown and Levine, 2008). Intrinsic LOC neurons also stain positive for CGRP and ChAT. In contrast, shell LOC neurons are reactive only to ChAT antibody (Kawai et al., 1985, Vetter et al., 1991).

1.4.1. Pathways of Olivocochlear Efferent Neurons

The axons of MOC neurons originating from cells located in the SOC can be divided into two populations based on their projections patterns. The first population is known as uncrossed MOC axons that innervate the ipsilateral cochlea. The second population is known as crossed MOC axons that innervate the contralateral cochlea. Upon reaching the floor of the fourth ventricle, the axons of uncrossed MOC neurons run ventrally to the facial nerve genua and continue through the vestibular nerve root, the vestibulocochlear anastomosis and the auditory vestibular nerve. In contrast, the axons of crossed MOC neurons travel medially and then cross the midline of the brainstem at the level of the floor of the fourth ventricle. The crossed MOC axons thereafter run ventrally to the facial genua and then follow a trajectory similar to that of the uncrossed MOC axons. The axons of LOC neurons originate from cells located in and around the LSO nucleus and follow the same pattern of projections of uncrossed MOC axons. Figure 1.5 is a schematic representation of the origin of OC neurons in the SOC of the cat and the course of their axons to the organ of Corti (Guinan, 2006).
Figure 1-5: Origin, course and termination of the olivocochlear (OC) neurons. (A) Transverse section of a cat brainstem showing OC neurons and the course of their axons to the right “ipsilateral cochlea”. Cell bodies of lateral olivocochlear (LOC) neurons are situated around and/or in the lateral superior olive (LSO), while cell bodies of the medial olivocochlear (MOC) neurons are situated in the medial regions of the superior olivary complex (SOC). Axons from LOC neurons (green) project mainly to the ipsilateral cochlea. Axons from MOC neurons either cross (blue) and project to the contralateral cochlea, forming the ipsilateral MOC reflex, or remain uncrossed (red) and project to the ipsilateral cochlea, forming the contralateral MOC reflex. The axons from LOC and MOC neurons form the olivocochlear bundle (OCB) (gold) are comprised of crossed olivocochlear bundles (COCB) and uncrossed olivocochlear bundle (UOCB). As the bundles proceed laterally, collateral branches are given off to the cochlear nucleus (CN) (yellow). The contralateral (red) and ipsilateral (blue) MOC reflexes are located in the right ear. (B) Simplified schematic diagram showing peripheral terminations of LOC neurons (green) on the dendrites of primary auditory nerve fibres beneath the inner hair cells (IHCs) and terminations of MOC neurons (blue) on outer hair cells (OHCs) (adapted from Guinan, 2006).

Previous studies in several species have established that the OCB synapses with two principal targets in the cochlea: OHCs (Engström, 1958; Bodian and Gucer, 1980) and dendrites of auditory nerve fibres beneath the IHCs (Smith, 1961; Liberman, 1980). Maison et al. (2003) cut the COCB, which is largely comprised of MOC fibres, and found that the efferent terminals on OHCs were partially lost, but the efferent terminals beneath the IHCs were unchanged. However, cutting the entire OCB within the vestibular nerve resulted in
the loss of all synaptic contacts not only with the OHCs but also with the IHCs (Smith and Rasmussen, 1963, Bodian and Gucer, 1980). This study suggests the crossing MOC axons innervate OHCs and the uncrossing LOC axons innervate IHCs. More importantly, using injections of the retrograde transporter horseradish peroxidase into the cochlea Warr and colleague (1979) have shown that LOC and MOC neurons make synaptic contact with two different types of cells in the organ of Corti (Warr and Guinan, 1979). The terminations of the LOC neurons are on the dendrites of auditory nerve fibres beneath the IHCs, whereas MOC neurons synapse directly with the OHCs of the organ of Corti (Figure 1.5B) (Robertson and Gummer, 1985, Liberman and Brown, 1986, Brown, 1987). The efferent innervation of the IHC region in cats was studied in more detail by Liberman and co-workers (1990, 1980). They found that the dendrites of auditory nerve fibres receive an average of 5-25 LOC synapses per fibre (Liberman et al., 1990). These synapses in the inner spiral bundle are formed by small *en passant* varicosities and a few terminal branches (Liberman, 1980). In the tunnel spiral bundle, LOC varicosities in some species such as cats (Liberman, 1980) and chinchilla (Iurato et al., 1978) might contact MOC branches on their way to the OHCs. In rats, Warr et al. (1997) injected an anterograde tracer inside and at the margins of the LSO to label intrinsic and shell neurons, respectively. This study observed that intrinsic neurons have short axons that terminate in separate dense arbors under the IHCs of the organ of Corti, and shell neurons have long axons that terminate in sparse branches beneath the IHCs (Warr et al., 1997). In cats, Guinan and colleagues (1984) using anterograde tracer injections found that injections into the medial region of the LSO (high characteristic frequency region) result in labelling the cochlea base, while injections into the lateral region of the LSO (low characteristic frequency region) result in labelling the cochlea apex. These results suggest that LOC neurons project to the cochlea in a mapping generally similar to the tonotopic mapping for the LSO (Guinan et al., 1984, Robertson et al., 1987a, Robertson et al., 1987c).

The terminations of the LOC neurons in the inner spiral bundle are also smaller than those of MOC neurons on the OHCs. In cats, the number of MOC terminals is greatest near the base of the cochlea and decreases apically (Guinan et al., 1984). The three rows of OHCs receive unequal innervation from the MOC terminals. The first row of OHCs receives the most MOC terminals, then the second row and lastly the third row (Liberman et al., 1990).
MOC synapses are formed by *en passant* varicosities which some of them in human contact type II auditory nerve fibres (Thiers et al., 2002). MOC neurons commonly project to a region of the cochlea where the auditory nerve fibres of the same characteristic frequency would be found (Robertson and Gummer, 1985, Liberman and Brown, 1986, Brown, 1989).

**1.4.2. Collateral Branches of Olivocochlear Efferent Neurons into the Cochlear Nucleus**

The OC neurons have been found to give off collateral branches to the vestibular nuclei (Brown, 1993) and the CN (White and Warr, 1983, Brown et al., 1988, Ryan et al., 1990). Only the collateral branches of OC neurons into the CN will be discussed further. These collateral branches have been widely studied in many species, including the rat, gerbil, guinea pig, mouse and cat. However, it is still unclear whether the collateral branches of OC neurons in the CN arise from the LOC or MOC neurons (White and Warr, 1983, Brown et al., 1988, Winter et al., 1989, Benson and Brown, 1990, Ryan et al., 1990, Brown, 1993, Benson et al., 1996, Horváth et al., 2000, Brown and Vetter, 2009). Injections of horseradish peroxidase into the cochlea of the rat have shown that axons of OC neurons leave the main efferent bundle at several points and enter the CN (White and Warr, 1983). These axons have been found to innervate the junction between the DCN and PVCN and the central region of the AVCN. This study, however, was unable to determine which classes of OC neurons send collateral branches to the CN.

Some neuroanatomical studies have suggested that both LOC and MOC axons send collateral branches to the VCN *en route* to the cochlea. Using injection of the retrograde neuronal tracers H-d-aspartate and H-nipecotic acid into the cochlea of the gerbil Ryan and colleagues (1990) reported that the central region of the VCN is mostly innervated by collaterals of the unmyelinated LOC neurons, whereas the myelinated MOC collaterals mostly innervated the peripheral parts of the VCN (Ryan et al., 1990). Horváth et al. (2000) by injecting diamidino yellow and fast blue into the cochlea and the VCN of the rat, found that shell LOC neurons on the margins of the LSO nucleus send collateral branches into the VCN, whereas intrinsic LOC neurons located within the LSO do not give off collateral branches to the CN. They also found that 80-100% of MOC neurons situated in the VNTB have axon collaterals that project into the VCN (Horváth et al., 2000).
Other studies, by contrast, have suggested that the collateral branches of OC neurons into the VCN in several species arise from MOC axons, but not from LOC axons (Brown et al., 1988, Brown and Benson, 1992, Brown, 1993). Injections of the retrograde tracer horseradish peroxidase into the cochlea of the cat, gerbil, and mouse have shown that only thick axons of MOC neurons form VCN branches (Brown et al., 1988, Brown and Benson, 1992). Brown (1993) studied the axon pathways of OC neurons and their branching patterns within the brainstem of the mouse and found that about two thirds of MOC axons send collateral branches into the VCN but LOC axons did not.

The number and proportions of MOC collateral branches projecting into the CN varies from one species to another. In the cat, mouse, gerbil and rat, the proportion of MOC collateral branches into the VCN ranges from 65-100% (Brown et al., 1988, Ryan et al., 1990, Brown and Benson, 1992, Horváth et al., 2000). However, in the guinea pig, only 3.5-9.9% of MOC axons are reported to give off collateral branches to the VCN (Winter et al., 1989). The small number of MOC collateral branches in the VCN of the guinea pig might be due to interspecies differences or the small size of injection in the CN in the paper of Winter et al. (1989) and hence the dye may not have been taken up by all collateral branches in the CN.

Most MOC axon collateral branches are located in the medial sheet of the VCN and in the dorsal edge of the granule cell lamina dividing the ventral from the dorsal subdivisions of the CN (Martin, 1981, Osen et al., 1984, Brown et al., 1988, Winter et al., 1989, Ryan et al., 1990, Brown, 1993). However, some collateral branches of the MOC axon are also found in the lateral edge of the VCN, in the central region of the VCN and in the subpeduncular granule region of the CN (Brown et al., 1988, Winter et al., 1989, Brown, 1993). In addition, a few collateral branches of MOC axons are also located in the strial corner of the DCN and in the superficial and deep cell layers of the DCN (Martin, 1981, Osen et al., 1984, Brown et al., 1988). Despite this large body of research suggesting the collateral branches of the MOC neurons innervate the CN, particularly the VCN, little is known about their synaptic targets.

**1.4.3. Targets of Medial Olivocochlear Collateral Branches in the Cochlear Nucleus**

The collateral branches MOC axons give rise to many swellings (varicosities) which are thought to be indicative of synaptic contacts (Benson and Brown, 1990, Brown and Benson,
The CN branches of the MOC system have been studied using extracellular injections into the spiral ganglion bundle (Benson and Brown, 1990, Benson et al., 1996). Previous ultrastructural investigations have shown that the postsynaptic targets of MOC collateral branches in the VCN are both varicose dendrites and large dendrites (Benson and Brown, 1990, Benson et al., 1996). The varicose dendrites are thought to arise from small cells and the large dendrites are thought to arise from multipolar cells. The synapses between MOC collateral branches and varicose dendrites are found in the granule cell lamina. The synapses between MOC collateral branches and the large dendrites are observed in the medial sheet near the auditory nerve root as well as in the granule cell lamina of the VCN (Benson et al., 1996). The synaptic terminals are asymmetric and have small round vesicles which suggest excitatory synapses (Benson and Brown, 1990, Brown and Benson, 1992, Benson et al., 1996).

The specific physiological action of the MOC collaterals to the CN is still unknown. A limited number of studies have been conducted to investigate the functional role of the MOC collaterals in the CN. Starr and Wernick (1968) studied the neuronal responses of the CN in the cat using electrical stimulation of the OCB at the floor of the fourth ventricle. Inhibitory and excitatory effects on spontaneous and tone-evoked activity of the CN cells were described in this experiment. However, because the OC neurons have peripheral and central actions, this study failed to illustrate the sole effects of MOC collateral branches in the CN (Starr and Wernick, 1968). In another experiment, activity of CN cells in the anaesthetised cats was recorded while applying direct electrical stimulation to the SOC (Comis and Whitfield, 1968, Comis, 1970). These authors found that after activation of the medial region of the SOC, all cells in the CN were excited, whereas following stimulation of a more lateral region all cells were inhibited (Comis and Whitfield, 1968, Comis, 1970). However, it is unclear whether these effects were due to activation of MOC neurons, which would still show a mixture of peripheral and central effects as in the studies by Comis and colleagues or to activation of the direct projection from the SOC to the CN (Spangler et al., 1987, Winter et al., 1989).

Other studies investigating the effect of activation of the MOC collaterals in CN have attempted to determine the precise targets of the MOC collaterals. This has led to two main hypotheses about which cell type is involved. One hypothesis states that T-stellate
(chopper) cells are innervated by MOC collateral branches. The other states that D-stellate (onset chopper) cells are targeted by the MOC collateral branches. An early in vitro mouse study by Fujino and Oertel (2001) suggested that T-stellate cells are innervated by MOC collateral branches (Fujino and Oertel, 2001). This study recorded VCN unit responses to cholinergic agonists in brain slices of the mouse CN and used intracellular injections of biocytin to validate the cell type based on the dorsal or ventral trajectory of their axons and on the shape of their dendritic arbors. They found that only two types of neuronal cells were responsive: the T-stellate cells and the bushy cells. They observed excitatory effects in 94% of T-stellate cells consistent with an excitatory action of MOC collaterals on chopper cells. The bushy cells were only weakly excited by the cholinergic agonists. No effects were observed in D-stellate cells. They concluded that MOC collateral branches make excitatory cholinergic connections with T-stellate (chopper) and bushy cells, rather than D-stellate (onset chopper) cells (Fujino and Oertel, 2001, Oertel and Fujino, 2001).

Fujino and Oertel’s study (2001), however, had a number of limitations. Firstly, the responses in T-stellate cells to cholinergic agonists were based on pharmacological manipulations which might not be enough to imply connectivity or reflect in vivo physiology. Secondly, the cholinergic action observed by Fujino and Oertel in the tissue mouse slices might also reflect the separate cholinergic input from the SOC to the VCN (Sherriff and Henderson, 1994). A direct cholinergic projection to the VCN has been shown from some nuclei of the SOC, including the VNTB, RPO and LNTB (Godfrey et al., 1990, Sherriff and Henderson, 1994, Warr and Beck, 1996). Finally, the VCN in species such as the gerbil, cat, rat and bat receives direct input from the collateral branches of LOC neurons which are possibly also cholinergic (Elverland, 1977, Spangler et al., 1987, Ryan et al., 1990, Vater and Feng, 1990, Horváth et al., 2000). In mice, however, nothing is known about this projection, and therefore more investigation is required.

Other investigators have used electrophysiological in vivo methods to investigate the targets of MOC collateral branches in the VCN of guinea pigs and rats (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). In contrast to Fujino and Oertel, their results suggest that MOC collateral branches make excitatory connections with onset chopper cells that correspond to D-stellate cells (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). Mulders et al. (2002, 2009) studied the effects of
MOC collateral branches in the CN of guinea pigs by electrically stimulating the OC axons at the floor of the fourth ventricle whilst recording the extracellular (Mulders et al., 2002) and intracellular (Mulders et al., 2009) responses of single neurons in the VCN. Even though the peripheral effects of the OC stimulation were eliminated using kanamycin or strychnine, Mulders and colleagues showed that acoustically driven firing rates in some onset chopper cells were higher after the OC system activation. Onset chopper cells in the VCN of guinea pigs also showed excitatory postsynaptic potentials (EPSPs) and spikes after single shocks delivered at the level of the fourth ventricle (Mulders et al., 2007). However, chopper and primary like cells, presumably corresponding to T-stellate and bushy cells respectively (Osen, 1969, Kiang, 1975, Smith and Rhode, 1989, Oertel et al., 1990), did not respond to either OC activation (using extracellular recordings) or showed inhibitory postsynaptic potentials (IPSPs) as measured using intracellular recordings (Mulders et al., 2002, Mulders et al., 2009). These results were confirmed in rat. Using intracellular recordings in the VCN of rats, Mulders et al. (2003) found that single shocks applied to the midline of the floor of the fourth ventricle at the level of the facial genua evoked EPSPs and action potentials in onset cells, most likely onset chopper cells (Mulders et al., 2003). However, activation of the MOC in this experiment was not confirmed independently (Mulders et al., 2003).

The work of Mulders et al. suggests that collateral branches of the descending MOC system do not provide direct excitatory input to the chopper cells but to the onset chopper cells (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). The cell bodies of onset chopper, D-stellate, cells are located on the border of the VCN and granule cell regions and the dendrites of the onset chopper cells extend into the granule regions of the CN where MOC synapses have been shown (Brown et al., 1988, Oertel et al., 1990, Ferragamo et al., 1998). However, the methods that were used to stimulate the MOC system in the studies of Mulders et al. have some limitations. For instance, the excitatory effects might be caused by antidromic activation (output axons) of the VCN cells, including D-stellate cells passing in the intermediate and dorsal acoustic striae (Mulders et al., 2009). However, the presence of EPSPs and the timing of D-stellate excitation in the work of Mulders et al. were consistent with the presence of orthodromic effects (activation of synaptic input) (Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009).
1.5. Potential Role of the Medial Olivocochlear Collateral Branches

1.5.1. Signal Detection in Noise

Based on physiological experiments, it appears that the MOC collaterals play an important role in improvement of the detection of signals in noisy background. As demonstrated in Figure 1.4, the output dynamic range of auditory primary afferent fibres in response to the tones in background noise is improved through the peripheral action of MOC system (the anti-masking effect (Winslow and Sachs, 1987)). This anti-masking effect depends on a suppression of amplification process of the cochlear OHCs and thus a full restoration of auditory primary afferent thresholds to the levels in quiet background is not achieved. In a noisy background with MOC axons activation, it has been found that responses of certain neuronal cell types in central auditory brain centres can be restored to the responses values in a quiet background (Mulders et al., 2008, Seluakumaran et al., 2008a). This result suggests the existence of additional central action into the known anti-masking effect of the peripheral terminations which may include an action of MOC collaterals.

1.5.2. Central Detection Adjustment

Mulders et al. (2002) studied the effects of MOC collaterals on the CN of guinea pigs by electrical stimulating the OC axons at the floor of the fourth ventricle whilst recording responses of single cells in the VCN. They found that responses of a number of cells in the CN did not alter with MOC activation in quiet background although reductions in responses of primary afferent neurons at the same sound frequencies were seen. Thus, it is possible to conclude that MOC collaterals play a role in re-calibrating central gain to compensate for changes that occur due to the action of the OC system at the auditory periphery. The properties of these cells in the CN in this study were not fully characterized and therefore, the specific function of these cells in the CN is still unclear (Mulders et al., 2002).

1.5.3. Modulating the Neural Hyperactivity in the Central Auditory Pathways

Several studies on animal models have shown spontaneous hyperactivity in the central auditory pathways after traumatizing the peripheral auditory receptor (Kaltenbach et al., 2000, Seki and Eggermont, 2003, Kaltenbach et al., 2004, Brozoski et al., 2007, Bauer et al.,
2008, Dong et al., 2009, Mulders and Robertson, 2009). Such hyperactivity has been related to the generation of tinnitus which is a phantom auditory perception commonly known as ringing in the ears (Kaltenbach et al., 2004, Brozoski et al., 2007, Bauer et al., 2008). A study using guinea pigs as a tinnitus animal model found that a suppression of the neural hyperactivity in the auditory midbrain was noticed after the stimulation of the MOC system (Mulders, 2006). Some of this suppression in the abnormal levels of the neural activity, hyperactivity, is mediated by reduction of input from the cochlea, but it seems that there is an additional central long-lasting action of the MOC stimulation that may be mediated by MOC collaterals. The so-called hyperactivity caused by acoustic trauma has also been found in the VCN of the brainstem (Vogler et al., 2011).

In a rat model of hyperactivity caused by unilateral acoustic injury, it has been found that growth-associated protein 43 (GAP-43) up-regulation, a marker for synaptic plasticity, most likely arises from the medial zones of the VCN (Kraus et al., 2011). This study showed that this up-regulation of GAP-43 is caused by a synaptic reorganisation in the VCN, possibly related to innervation by MOC collaterals. This reorganisation is inversely related to the severity of tinnitus. Kraus et al. (2011) suggested that a possible inhibitory effect of the MOC collaterals might be involved in the reduction of the tinnitus-related hyperactivity.

Cope et al. (2011) investigated the effect of contralateral external acoustic noise on the severity of tinnitus in patients who had unilateral tinnitus. They demonstrated that the effect of contralateral sound was highly dependent on whether the vestibular nerve on the site of tinnitus perception was damaged or not. This damage affects the peripheral projection of MOC to the cochlea, but leaves their collaterals to the CN intact (Cope et al., 2011). The MOC neurons activate when contralateral external acoustic noise is delivered to the side where the vestibular nerve is intact (Robertson and Gummer, 1985, Guinan et al., 2003). These studies, therefore, suggest that the effects observed might be due to the disturbance of balance between the peripheral and central actions of the MOC system. Physiological evidence supports a role of the MOC collaterals in modulating the neural hyperactivity in central pathways caused by peripheral cochlear damage.
1.6. The Specific Aims of the Thesis

Available evidence based on anatomical studies found that collateral branches of the MOC neurons make synaptic contacts with multipolar cells in the CN. These multipolar cells, however, consist of two distinct neuronal populations: T-stellate (chopper) cells, thought to project to the IC and D-stellate (onset chopper) cells, thought to project to the contralateral CN. Each of these neuronal subtypes serves a variety of roles in auditory processing. To understand the physiological properties and functional roles of the MOC collateral branches, it is essential to determine their exact target cells in the CN. At present the targets of MOC collateral branches in the CN are still unclear since a conflict exists between results obtained from *in vivo* electrophysiological studies (Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009) and *in vitro* pharmacological studies (Fujino and Oertel, 2001). Therefore, the focal objective of this thesis is to investigate the anatomical relationship between MOC collateral branches and T-stellate and D-stellate cells. For this purpose, the proposed research will seek to address the following questions:

1. Is the pattern of innervation of the CN by MOC neurons the same in rats, guinea pigs and mice?

2. Do MOC collateral branches show anatomical contacts with CN cells projecting to the IC (presumed T-stellate/chopper cells)?

3. Do MOC collateral branches show anatomical contacts with cells projecting to the contralateral CN (presumed D-stellate/onset chopper cells)?

4. Do MOC collateral branches show anatomical contacts exclusively with glycinergic cells projecting to the contralateral CN (presumed D-stellate/onset chopper cells)?
Chapter 2
General Methods

2.1. Overview of Experimental Design in Guinea Pigs and Rats

Double-labelling experiments were employed to identify which neural cell types in the VCN (T-stellate cells or D-stellate cells) receive synaptic innervation from the collateral branches of MOC axons. These experiments involved the injection of tracers into the IC, CN and at the floor of the fourth ventricle in guinea pigs and rats (Figure 2.1).

T-stellate cells project directly to the IC via the trapezoid body. As no other cell type in the PVCN and AVCN has these projections (see Chapter 1, Section 1.2.2.3.), retrograde tracer injections into the CNIC in guinea pigs and rats should label multipolar (T-stellate) cells in the VCN. In contrast, D-stellate cells project dorsalward via the intermediate acoustic stria to the contralateral PVCN and AVCN (see Chapter 1, Section 1.2.2.3.). Therefore, retrograde tracer injections into the VCN in guinea pigs and rats should label multipolar (D-stellate) cells in the contralateral VCN.

The axons of MOC neurons travel dorsomedially from the medial, ventral and rostral regions of the SOC, form a tight bundle at the midline of the brainstem at the level of the floor of the fourth ventricle and then project to the cochlea. Axons of MOC neurons en route to the cochlea give off collateral branches to the CN (see Chapter 1, Section 1.4.2.). As MOC axons are grouped in a tight bundle at the centre of the floor of the fourth ventricle, between the facial genua, we expected the injections of biocytin in this location to label the MOC neurons and their axons and collateral branches in the VCN in guinea pigs and rats. The appositions of retrogradely labelled VCN cells and anterogradely labelled MOC axonal varicosities in the VCN were then assessed as an indication of innervation.

2.2. Overview of Experimental Design in Mice

As described above for guinea pigs and rats, the neuronal tracer, biocytin, was also injected at the centre of the floor of the fourth in wild-type and transgenic mice. The latter animals express EGFP under the control of the promoter of the GlyT2 gene, which is a marker for glycineric (presumably including D-stellate) cells (Zeilhofer et al., 2005). The appositions of
EGFP (D-stellate) labelled cells and anterogradely labelled MOC axonal varicosities in the VCN were then assessed as an indication of innervation.

Figure 2-1: Schematic representation of experimental set-up. 1) FG injection into the central nucleus of the inferior colliculus (CNIC) (as indicated by the blue circular area) is expected to label multipolar neurons (T-stellate cells) in the posterior (PVCN) and anterior (AVCN) ventral cochlear nuclei. 2) FG injection into the VCN (as illustrated by the red circular area) is expected to label multipolar neurons (D-stellate cells) in the PVCN and AVCN nuclei. Retrograde projection is indicated by the blue and red lines and labelled cell bodies in the VCN are indicated with a star. 3) Biocytin injection at the floor of the fourth ventricle (as indicated by the green circular area) is expected to label collateral branches of medial olivocochlear (MOC) neurons in the VCN. Anterograde projection is indicated by the green lines.
2.3. Animal Subjects

Experiments were performed on pigmented guinea pigs (250-450g) obtained from the University of Western Australia (Large Animal Facility), Wistar outbred rats (280-370g) obtained from the Animal Resource Centre (ARC), wild-type mice (23-35g) obtained from the ARC and transgenic mice from the Garvan Institute of Medical Research. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia. The procedures for the use and care of animals reported in this project were regulated and approved by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1175 for guinea pigs and rats and RA/3/100/1402 for mice).

2.4. Retrograde Tracer Injections in Guinea Pigs and Rats

2.4.1. Anaesthesia

Guinea pigs were pre-medicated with a subcutaneous (s.c.) injection of 0.1 ml atropine sulphate (0.6mg/ml, Apex Laboratories) and anaesthetised with 1ml/kg intraperitoneal (i.p.) Pamlin (diazepam 5mg/kg, Ceva) and 1ml/kg intramuscular (i.m.) Hypnorm (fentanyl citrate 0.315mg/ml, fluanisone 10mg/ml, Vecta Pharma). Rats were anaesthetised with a single i.p. injection of 60mg/kg Pentobarbitone (pentobarbitone sodium, 60mg/ml, Ilium).

In both guinea pigs and rats, depth of anaesthesia was confirmed by absence of foot withdrawal reflex. The fur on the skull was then shaved and 0.1ml Lignocaine (20mg/ml, Illium) was injected s.c. at the site where the incision was to be made. If foot withdrawal returned during surgery, 0.1ml of Hypnorm was given to the guinea pigs and a quarter dose of Pentobarbitone was given to the rats.

2.4.2. Surgical Procedures

Once full depth of anaesthesia was achieved, the animals were rested on a thermostatically controlled heating pad and covered with a blanket to maintain their body temperature at 37-38°C throughout surgery. Lacrilube eye ointment was applied to prevent corneal drying during the surgery. The animal’s head was placed in a stereotaxic frame. A small incision was made along the midline of the scalp. Tissues and muscles attached to the frontal and
parietal bones were pushed aside to expose the skull surface and surgical hooks were used to keep the area open. Two anatomical landmarks on the skull, bregma and lambda, were then identified. These landmarks were set to the same dorsal-ventral coordinates by adjusting the head position until the z coordinates (i.e. the dorsal-ventral coordinates) of bregma and lambda were equal. An interaural line (horizontal line or x-axis) was drawn between the two ears and was used as the anterior-posterior reference point. The midline suture along the skull (vertical line or y-axis) was used as the medial-lateral reference point and bregma was used as the dorsal-ventral reference point.

For the guinea pigs, the IC and VCN were located using stereotaxic coordinates from a guinea pig stereotaxic atlas (Rapisarda and Bacchelli, 1977). The correct location was confirmed by recording the nerve cell responses to sound (described in Section 2.4.3.). For rats, the IC and VCN were located using stereotaxic coordinates from the atlas of the rat brain (Paxinos and Watson, 1982).

After determining the site of injection using the stereotaxic coordinates, a dental drill was used to make a small craniotomy in the skull to expose the brain. The bone flaps were retained to return back in place after the retrograde tracer injections. Meninges were pierced with a fine hypodermic needle to expose the cerebrum and cerebellum overlying the IC and VCN, respectively.

2.4.3. Neural Recording and Stimulus Generation and Presentation in Guinea Pigs only

Injection pipettes (GC 120-15, Clark Electromechanical Instrument) were pulled using a P-87 micropipette puller (Sutter Instrument Co.). These pulled pipettes were used as recording electrodes (for guinea pigs only) as well as injection pipettes. The pipettes were shortened manually using fine ophthalmic scissors under visual control with a microscope (Zeiss West Germany) at 3.2x magnification to obtain a diameter ranging from 100-120µm. Care was taken to ensure the pipettes had angled tips to minimize the chances of blockage. The injection pipettes were filled with FG solution (Fluorogold, Fluorochrome LLC, U.S.A.; 8% solution dissolved in saline (0.9% sodium chloride)), connected to the microinjection apparatus (BAB-200, Kation Scientific), and lowered gently into the right IC and VCN injection sites.
To record nerve cell responses to sound, silver wire was placed in the pipette and reference and earth silver wires were placed in the neck muscles. The recorded signal was amplified (x1000) using an isolated bio-amplifier (Model ISO-80, WPI), filtered (300 Hz-3 kHz) and displayed on a computer monitor via an analog/digital interface (ADI-9 DS, RME Intelligent Audio Solution) and custom software (Neurosound MI Lloyd). The activity of multi-neuron cluster at different depths of the IC and VCN was recorded and used to define the site of the retrograde tracer injections into the IC and VCN (Rose et al., 1959, Rose et al., 1960, Clopton and Winfield, 1973, Popelár and Syka, 1982, Stiebler and Ehret, 1985, Romand and Ehret, 1990, Malmierca et al., 1995).

Sound stimuli were delivered by means of a closed sound system. All sound stimuli were delivered monaurally to the external auditory meatus (contralateral to the injected IC and ipsilateral to the injected CN) via a hollow ear bar, using a speaker consisting of a 1/2-inch condenser microphone driven in reverse (Bruel and Kjaer, model 4134). The stimuli were pure tones or broadband noise of 50ms duration and a repetition rate of 2/s for IC and 4/s for VCN, generated by a DIGI 96 soundcard connected via optical cable to an analog/digital interface (ADI-9 DS, RME Intelligent Audio Solution). The computer interface was driven by a custom-made program (Neurosound, MI Lloyd).

2.4.4. Fluorogold Tracer Injections

Injections of FG tracers were made at two sites into the IC and three sites into the CN within each animals in order to maximise the number of retrogradely labelled cells in the VCN (see results Chapters 4 and 5). The tracer was iontophoretically delivered by passing a 10µA-13µA positive current, with a 5 second pulse on/off cycle for 10 minutes (BAB-200, Kation Scientific). Following injection of the tracer, the electrode was left in place for an additional 5 minutes before withdrawal to avoid pulling the FG solution back up the pipette track. The bone flap was then replaced, the scalp was sutured, and the animals were rested on a thermostatically controlled heating pad. After full recovery from anaesthesia, guinea pigs and rats were returned to the animal holding facility. In the holding facility, animals were monitored daily for swelling or exudate around the incisions and weighed daily for 5-7 days after surgery.
2.5. Bidirectional Tracer Injections in Guinea Pigs, Rats and Mice

2.5.1. Anaesthesia

Five to seven days after FG injection guinea pigs were injected s.c. with 0.1ml atropine sulphate and anaesthetised with 30mg/kg i.p. Pentobarbitone and 0.15ml i.m. Hypnorm. Rats were injected with 60mg/kg Pentobarbitone i.p. and 0.1ml atropine sulphate s.c. The mice (wild-type and transgenic) were anaesthetised by i.p injection of 20% urethane (0.085ml/10g, Sigma).

In both the guinea pigs and rats, either the right or left animals’ hind and front paws were each pierced with a 27G needle which were used as electrodes for electrocardiogram recording. The recorded signal was amplified (x1000) using an isolated bio-amplifier (Model ISO-80, WPI) and displayed on a digital oscilloscope (Rigol, Model-DS1052E) to monitor the heart rate throughout surgery. To maintain deep surgical anaesthesia throughout the experiment, guinea pigs were injected with 0.15ml Hypnorm every hour and a half dose of Pentobarbitone every two hours. Rats were given a quarter dose of Pentobarbitone and the mice were injected with 0.01ml/10g urethane if foot withdrawal returned.

2.5.2. Electrical Stimulation of Facial Nerve and Biocytin Tracer Injections

The location of the MOC axons in guinea pigs was mapped using a technique described by Seluakumaran et al. (2008a). This technique is based on the fact that axons of MOC neurons are situated close to the facial nerve axons as described by Aschoff and Ostwald (1987).

In the guinea pigs, a tracheostomy was performed and the animals were artificially ventilated on carbogen (95% oxygen and 5% carbon dioxide). The guinea pigs were then placed on a thermostatically controlled heating pad and covered with a blanket. Ear bars were then positioned and, posterior to the interaural line, a large craniotomy (3mm x 4mm) was drilled in the skull to expose the brain. The overlying meninges were pierced with a sterile fine hypodermic needle to expose the cerebellum and the cerebellum was partially aspirated by gentle suction using a pipette connected to a vacuum pump to enable visualisation of the midline of the floor of the fourth ventricle. Stimulating electrodes (platinum-iridium concentric bipolar electrodes with a tip diameter between 3 and 4µm,
World Precision Instruments, USA) connected to an isolated stimulator output (A-M System, Model-2100) were then aimed toward the midline of the brain stem to map the thresholds for facial nerve activation assessed by whisker twitch. To achieve this, single shocks (0.1ms duration) were delivered at a rate of 1/s to the midline of the floor of the fourth ventricle. Once facial twitch was evoked, the shock amplitude was gradually reduced until the facial twitch stopped. The depth of the stimulating electrode and threshold voltage were recorded. Similar recordings were performed at similar depth whilst moving the stimulating electrode along the antero-posterior axis along the midline of the fourth ventricle. The bidirectional tracer was injected at the lowest threshold voltage which previously studies have shown to be the location of the MOC axons between the facial genua (Seluakumaran et al., 2008a).

The injection pipettes, as described previously in Section 2.4.3., were pulled and shortened to obtain a tip diameter ranging from 200-230µm. They were then filled with biocytin hydrochloride solution (Sigma, U.S.A.; 1% in 150mM KCl) and slowly inserted at the lowest threshold point that evoked the facial twitch. Bidirectional tracer was then iontophoretically injected by passing a 10µA-14µA positive current, with a 5 second pulse on/off cycle for 3 hours (BAB-200, Kation Scientific).

In the rats and mice, stereotaxic surgery was performed as described in Section 2.4.2., without modification. The site of biocytin injections at the floor of the fourth ventricle in the rats and mice was determined using stereotaxic coordinates (Paxinos and Watson, 1982). The duration of biocytin injections at the floor of the fourth ventricle was 2 hours. In all animals, the pipette was left in place for an additional 60 minutes after biocytin injection stopped to allow for sufficient transport of tracer, and then slowly removed (Baashar et al., 2015).

2.6. Histological Processing

After removal of the injection pipette, the animals were euthanized with an i.p. injection of 0.3ml Lethabarb (pentobarbitone sodium 325 mg/ml, Virbac Animal Health, Australia). The guinea pigs and rats were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH7.4). The mice were injected with
0.05ml of Heparin into the left ventricle and perfused with 5ml of 0.2M PB followed by 60ml of 4% paraformaldehyde in 0.1M PB (pH7.4). In the guinea pigs, rats and mice, brains were dissected from the skull and post-fixed in the same fixative overnight at 4°C. The next day, brains were cryoprotected in 30% sucrose solution in 0.1M PB and left overnight at 4°C. They were then frozen, sectioned in the transverse plane (60µm thickness) with a freezing microtome (Kryomat 1703, Leitz, for the guinea pigs and rats) or (40µm thickness) with a cryostat (Leica CM3050 S, for the mice) and collected in 0.1M PB.

For the double labelling experiments (biocytin and FG or EGFP), serial sections were cut covering the full extent of the CN, SOC and IC. Every 7th section was used for counterstaining with toluidine blue and the other sections were stained free-floating for biocytin in order to visualise the biocytin-labelled MOC neurons and their axons and collateral branches in the VCN, and FG or EGFP. During staining, all sections were incubated in glass vials and agitated on a shaker. Sections were rinsed 3 times in 0.1M PB and then incubated for 10 minutes in 3% hydrogen peroxide (H₂O₂) in methanol to eliminate endogenous peroxidase activity. The sections were then rinsed again in 0.1M PB 3 times and incubated in 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.1M PB for 60 minutes at room temperature. Sections were then rinsed 3 times in 0.1M PB and incubated at room temperature in ABC solution (1:800, Elite Pk-6100 Standard Vectastain ABC Kit, Vector Laboratories) for 2 hours. After 3 rinses with 0.1M PB, the sections were then reacted for 6 minutes with DAB solution (0.02% 3,3’-diaminobenzidine (DAB), Sigma) in 0.3% Tris Buffer with 0.2% ammonium nickel sulphate (Univar) and 30µl of 3% H₂O₂ at pH7.6. This procedure resulted in blue-black biocytin labelling of the MOC neurons, MOC axons and collateral branches in the VCN. Collateral branches of MOC neurons in the VCN were visualised using DAB amplification as described above, and immuno-labelling was used to stain FG (guinea pigs and rats) or EGFP (mice) labelled cells in the VCN.

For the FG experiments, the same sections, which were stained for biocytin, were then rinsed 5 times in 0.1M PB and incubated in a solution of 5% goat serum (Sigma), 0.1% BSA, 0.3% Triton X-100 and (1:1000 or 1:500 rabbit anti-FG, Fluorochrome) in 0.1M PB for two days at 4°C. After 3 rinses with 0.1M PB, the sections were incubated for 90 minutes at room temperature in a solution consisting of 5% goat serum, 0.1% BSA, 0.3% Triton X-100
and (1:200) biotinylated goat anti-rabbit secondary antibody (Sigma) in 0.1M PB. Sections were then rinsed 3 times in 0.1M PB and incubated at room temperature in ABC solution (1:800, Elite Pk-6100 Standard Vectastain ABC Kit, Vector Laboratories) for 2 hours. After 3 rinses with 0.1M PB, the sections were reacted for 10 minutes with DAB solution (0.02% 3,3’-DAB, Sigma) in 0.3% Tris Buffer and 30µl of 3% H₂O₂ at pH7.6. This procedure resulted in red-brown FG labelled cells in the VCN. Finally, the sections were rinsed 5 times in 0.1M PB.

For the EGFP experiments, the same sections, which were stained for biocytin, were then rinsed 5 times in 0.1M PB and incubated in a solution of 5% goat serum (Sigma), 0.1% BSA, 0.3% Triton X-100 and (1:1500 rabbit anti-GFP, Abcam) in 0.1M PB for two days at 4°C. After 3 rinses with 0.1M PB, the sections were incubated for 90 minutes at room temperature in a solution consisting of 5% goat serum, 0.1% BSA, 0.3% Triton X-100 and (1:500) biotinylated goat anti-rabbit secondary antibody (Sigma) in 0.1M PB. Sections were then rinsed 3 times in 0.1M PB and incubated at room temperature in ABC solution (1:800, Elite Pk-6100 Standard Vectastain ABC Kit, Vector Laboratories) for 2 hours. After 3 rinses with 0.1M PB, the sections were reacted for 10 minutes with DAB solution (0.02% 3,3’-DAB, Sigma) in 0.3% Tris Buffer and 30µl of 3% H₂O₂ at pH7.6. This procedure resulted in red-brown GFP labelled cells in the VCN. Finally, the sections were rinsed 5 times in 0.1M PB.

As dendritic morphology was not optimal after immuno-labelling of the FG using DAB amplification, attempts were made to improve the FG labelling. Firstly the tip size of the pipettes was increased from 55 to 100µm, but this did not result in an increase in retrograde labelling. Next, the animal survival time after FG injection into the CNIC was increased from 7 to 14 days but the same problem occurred with labelling cells in the VCN. Increasing the incubation time of the DAB reaction resulted in an increase in the background staining, making FG labelled cells in the VCN difficult to distinguish. Increasing the concentration of the primary antibody (antibody to FG) from 1:1000 to 1:500 also resulted in an increase in the intensity of staining of the cells in the VCN, but not to levels that sufficiently stained the dendritic trees of the FG labelled cells in the VCN (Figure 2.2). The limited success with immunostaining of the dendrites of FG labelled cells in the CN, led to the decision to choose alternative methods for analysis. Because FG fluorescence gave
good revelation of somatic and dendritic morphology, direct observation of FG fluorescence was used in combination with the usual method of biocytin processing to generate a dark reaction product in MOC axons and collaterals. Using this method, it was found that biocytin-labelled processes could be observed at the same time as FG fluorescence. Therefore, fluorescence techniques were used to identify the FG labelled cells, immunohistochemical methods were used to identify the EGFP and histochemical techniques were used to identify the biocytin-labelled MOC neurons, their axons and collateral branches.

Figure 2-2: Photomicrographs of retrograde labelling in the cochlear nucleus (CN) in guinea pigs using immuno-labelling methods. The first column showing FG labelled cells in A) the dorsal (DCN) and C) the ventral (VCN). FG antibody in A and C was 1:1000. The second column showing FG labelled cells in B) the DCN and D) the VCN. Antibody to FG in B and D was 1:500. Scale bars in A-D = 50µm.

The reference sections (every 7th section) were counterstained for identification of cytoarchitectural borders. These sections were mounted immediately onto gelatin-coated slides (Menzel-Gläzer) and allowed to air dry overnight. The sections were then stained with toluidine blue, dehydrated in a graded series of ethanol, cleared in xylene and coverslipped. Sections that had undergone histochemical processing for biocytin were mounted and coverslipped using the same protocol without the toluidine blue staining step.
2.7. Histological Analysis

The location of the cytoarchitectonic borders in different series of the CN was verified by comparison with the adjacent toluidine blue stained sections. The different subdivisions were defined using the descriptions of Harrison and Warr (1962), Harrison and Irving (1966a), Harrison and Irving (1966b), Mugnaini et al. (1980), Webster and Trune (1982), Ryugo and Willard (1985) and Hackney et al. (1990). As shown in Figures 2.3, 2.4 and 2.5, the CN is composed of two nuclei, the DCN and the VCN. The DCN is situated caudal and dorsal to the VCN. It is a laminar structure and is separated from the VCN by the granule cell region. The VCN, on the other hand, is not a laminar structure as the DCN and is divided further by the bifurcation of the auditory nerve root into two principal subdivisions, the PVCN and the AVCN.
Figure 2-3: A series of toluidine blue-counterstained transverse sections (60µm thick) illustrating the location of the cytoarchitectonic borders of the dorsal (DCN), ventral (VCN) cochlear nuclei and the granule cell layer (GCL) in guinea pig. A is the most caudal section and I is the most rostral section. The distance between sections is = 420µm. Medial (M) and dorsal (D) in A is also for B-I. Scale bars in A-I = 1000µm.
Figure 2-4: A series of toluidine blue-counterstained transverse sections (60µm thick) illustrating the location of the cytoarchitectonic borders of the dorsal (DCN), ventral (VCN) cochlear nuclei and the granule cell layer (GCL) in rat. A is the most caudal section and F is the most rostral section. The distance between sections is = 420µm. Medial (M) and dorsal (D) in A is also for B-F. Scale bars in A-F = 1000µm.
Figure 2-5: A series of toluidine blue-counterstained transverse sections (40µm thick) illustrating the location of the cytoarchitectonic borders of the dorsal (DCN), ventral (VCN) cochlear nuclei and the granule cell layer (GCL) in mouse. A is the most caudal section and F is the most rostral section. The distance between sections is = 280µm. Medial (M) and dorsal (D) in A is also for B-F. Scale bars in A-F = 500µm.

The subdivisions of the SOC were similarly based on the toluidine blue stained series with reference to previous work by others. As shown in Figures 2.6, 2.7 and 2.8, the SOC is composed of three primary nuclei, the LSO, MSO and MNTB, surrounded by a number of less distinct cell groups, collectively referred to as the periolivary nuclei. The LSO is situated in the lateral and dorsal regions of the SOC. The MSO is a narrow band of neurons situated in the central region of the SOC. The MNTB is situated in the most medial region of the SOC (Ollo and Schwartz, 1979, Osen et al., 1984, Faye-Lund, 1986, Schofield and Cant, 1991).
The periolivary nuclei are typically named according to their location with regard to the MSO, LSO and MNTB (Figures 2.6, 2.7 and 2.8). The VNTB is located in the region lateral to the MNTB and ventral to the MSO, while the LNTB lies in the region just ventral to the LSO. The region dorsomedial to the MSO is assigned as the SPN which probably corresponds to the DMPO (Ollo and Schwartz, 1979, Osen et al., 1984, Faye-Lund, 1986, Schofield and Cant, 1991). The RPO is defined as the region directly rostral to the LSO and before the VNLL.

Figure 2-6: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of (A) the ventral nucleus of the trapezoid body (VNTB), the dorsomedial periolivary nucleus (DMPO) and (B) the rostral periolivary nucleus (RPO) in guinea pig. The distance between sections is = 1680µm. Medial (M) and dorsal (D) in A is also for B. Scale bars in A and B = 1000µm.
Figure 2-7: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of (A) the ventral nucleus of the trapezoid body (VNTB) and (B) the rostral periolivary nucleus (RPO) in rat. The distance between sections is = 1260µm. Medial (M) and dorsal (D) in A is also for B. Scale bars in A and B = 1000µm.
Figure 2-8: Toluidine blue-counterstained transverse sections showing the location of the cytoarchitectonic borders of (A) the ventral nucleus of the trapezoid body (VNTB) and (B) the rostral periolivary nucleus (RPO) in mouse. The distance between sections is = 840µm. Medial (M) and dorsal (D) in A is also for B. Scale bars in A and B = 1000µm.

The location of the main IC subdivisions was distinguished according to the criteria established by others (Faye-Lund and Osen, 1985, Ryugo and Willard, 1985, Meininger et al., 1986, Malmierca et al., 1995). The IC is composed of three nuclei, the central nucleus (CNIC), dorsal cortex (DCIC) and external cortex (ECIC). The CNIC is bordered by the DCIC dorsally and surrounded by the ECIC ventrally and laterally.
FG and EGFP labelled cells in the VCN were counted and photographed using a fluorescent microscope (Nikon Eclipse 80i, Japan) equipped with the UV-2A fluorescence filter (330–380nm excitation and 420nm barrier filters) and integrated Digital Sight Camera using NIS Elements Advanced Research software (Nikon version 3). When counting, a multipolar cell was defined as a cell possessing three or more dendrites extending from the soma in different directions (Figure 2.9). Counting was performed in all biocytin stained sections (6 out of every 7 sections). The size of the somata of FG and EGFP labelled cells in the CN and biocytin-labelled cells in VNTB, DMPO and RPO was estimated using NIS Elements Advanced Research software (Nikon version 3). The soma profile was manually traced excluding the dendrites at the point of their emergence from the soma (Figure 2.9).

Figure 2-9: Photomicrographs showing FG labelled cells in the ventral cochlear nucleus. (A and B) FG labelled multipolar cells with more than two dendrites emanating from the cell body in different directions as indicated by yellow arrows. One of these dendrites has an additional two branches as indicated by blue arrows. The cell body size of this multipolar cell was estimated by manually tracing the cell body profile excluding the dendrites at the point of their emergence from the cell body as indicated by the dashed circle. (C) A FG labelled cell with one dendrite. (D) A FG labelled cell with two dendrites. Counting was performed only in FG labelled cells that showed more than two dendrites as in A and B. Scale bars in A-D = 50µm.
The number of labelled MOC neurons and *en passant* and terminal varicosities on the collateral branches in the VCN were also counted. Varicosities were defined as enlargements along or at the tips of the collateral branches more than 60% larger than the diameter of the MOC axons (Baashar et al., 2015). Drawings from the sections were made using a Zeiss Standard 20 Microscope equipped with a drawing tube. Photoshop (Adobe Photoshop CS5) and Microsoft Photo Editor were used to adjust the brightness, colour balance and contrast of the photographs. Neuron counts were corrected for the fact that only 6 out of 7 sections were counted and for double-counting using the Abercrombie correction

\[ N = \frac{T}{T+D} \times n \]

where \( N \) = the total number of cells, \( T \) = thickness of the section, \( D \) = the average cell diameter, and \( n \) = number of cells counted (Abercrombie, 1946).

The criterion for contact was the existence of a varicosity of a MOC axon touching a cell body or dendrite of a FG or EGFP cell in the VCN. Close appositions were observed using either a 20x objective or a 40x oil-immersion objective. Once putative close appositions were identified, z-stack images were obtained using a Nikon Inverted Eclipse Ti connected to Roper Scientific Cool SNAP EZ camera using NIS elements Advanced Research software (Nikon version 4.13). The z distance between successive images in each stack ranged between 0.2-0.5µm. Each image in the z-stack was inspected to verify whether or not there was possible close apposition of FG or EGFP labelled cell and biocytin-labelled MOC varicosity. They were then merged to produce an overlay of full depth separately scalable photographs.

Putative close appositions were counted only if FG or EGFP labelled cells and anterograde labelled MOC axonal varicosities were both in sharp focus in the same slice of the z-stack. The merged z-stack image in Figure 2.10A shows an example of putative close apposition of an FG labelled cell and MOC axonal varicosity. In this example, many slices had both structures in focus (Figure 2.10B) although in some slices both were unfocused (Figure 2.10C). Therefore, this close apposition was counted. The merged z-stack image in Figure 11A also shows an example of apparently close apposition of FG labelled cell and biocytin-labelled MOC axonal varicosity but this merged image is misleading. In this example, none of
the slices had both structures in focus (Figure 2.11B-C). This close apposition, therefore, was not counted, as both structures were not in sharp focus together in any of the individual z-stack slices. The number of close appositions was counted in all biocytin-labelled sections.

Figure 2-10: Photomicrographs showing an example of putative close apposition of an FG labelled cell and medial olivocochlear (MOC) axonal varicosity. (A) The merged image of the z-stack showing example of putative close apposition of retrogradely labelled cell and anterogradely labelled MOC axonal varicosity. (B) FG labelled cell and MOC axonal varicosity are at a similar level of focus level. (C) Both structures are out of focus together. A putative close apposition was counted only if FG labelled cell and MOC axonal varicosity were focused at the same slice of the z-stack images as illustrated in this example. Scale bars in A-C = 100µm.
Figure 2-11: Photomicrographs showing a misleading example of putative close apposition of an FG labelled cell and medial olivocochlear (MOC) axonal varicosity. (A) The merged image of the z-stack. (B) FG labelled cell is focused in a slice in which MOC axonal varicosity was not. (C) MOC axonal varicosity is focused in a slice in which FG labelled cell was not. Counting was not performed in such example as FG labelled cell and MOC axonal varicosity are not focused in the same slice of the z-stack. Scale bars in A-C = 100µm.
3.1. Introduction

The aim of this study was to label MOC axons and their collateral branches in the CN. To label as many as possible of the MOC neurons, their axons and collateral branches in the CN, the neuronal tracer biocytin was injected at the centre of the floor of the fourth ventricle where MOC axons are grouped in a tight bundle between the facial genua. For more details of this labelling method see Chapter 2.

3.2. Results

3.2.1. Locations and Characteristics of Biocytin Injection Sites

Injections of biocytin were successful in labelling MOC axons in 10 guinea pigs, 14 rats and 1 mouse. In the 10 successfully labelled guinea pigs, the size of the biocytin injections varied from 225-607µm (mean = 384, SD = 138µm) in the dorso-ventral aspect, from 154-430µm (mean = 286, SD = 106µm) in the medio-lateral aspect and from 360-840µm (mean = 600, SD = 164µm) in the rostro-caudal aspect. In the 14 successfully labelled rats, the size of the biocytin injections ranged from 175-679µm (mean = 369, SD = 144µm) in the dorso-ventral aspect, from 168-399µm (mean = 269, SD = 55µm) in the medio-lateral aspect and from 360-600µm (mean = 420, SD = 70µm) in the rostro-caudal aspect. In the one successfully labelled mouse, the size of the biocytin injection was 432µm in the dorso-ventral aspect, 176µm in the medio-lateral aspect and 360µm in the rostro-caudal aspect. The floor of the fourth ventricle was hard to distinguish due to excessive damage from the injections. However, the injection sites could be easily identified (Figures 3.1A, 3.2A and 3.3A).

The biocytin injections were not successful in 48 guinea pigs, 53 rats and 24 mice. In these animals, the injections were placed at the midline but they were more dorsal or caudal and ventral to the facial genua. The caudal and ventral injections resulted in labelling of axons of the dorsal acoustic stria as evidenced of the existence of retrogradely labelled fusiform and giant cells in the DCN (Figures 3.1B, 3.2B and 3.3B) (Fernandez and Karapas, 1967, Adams
and Warr, 1976, Adams, 1979). However, the caudal and ventral injections did not result in labelling any of multipolar (D-stellate) cells in the PVCN and AVCN, confirming the fact that the axons of multipolar (D-stellate) cells do not leave the CN via the dorsal acoustic stria. These animals will not be described further in the present study and the following description is restricted to the results obtained from animals in which MOC axons were successfully labelled.

Figure 3-1: Photomicrographs showing the results of successful (A and C-I) and unsuccessful (B) injections in guinea pig. (A) An example of a successful injection of the bidirectional tracer biocytin at the centre of the floor of the fourth ventricle. (B) Labelled cells in the dorsal cochlear nucleus (DCN) as a result of labelling axons of the dorsal acoustic stria after unsuccessful injections, in this case caudal and ventral to facial genua (different animal than shown in A). (C) Retrogradely labelled cells in the ventral nucleus of the trapezoid body (VNTB), (D) the rostral periolivary (RPO) nucleus and (E) the dorsomedial periolivary (DMPO) nucleus. (F) Absence of labelled cells in the nucleus of lateral superior olive (LSO) following injections of the bidirectional tracer biocytin at the centre of the floor of the fourth ventricle. (G, H and I) High magnification images of medial olivocochlear (MOC) neurons with their extensive dendrites in (G) the VNTB, (H) the RPO and (I) the DMPO. Scale bars in A-F = 100µm, in G-I = 50µm.
Figure 3-2: Photomicrographs showing the results of successful (A and C-H) and unsuccessful (B) injections in rat. (A) An example of a successful injection of the bidirectional tracer biocytin at the centre of the floor of the fourth ventricle. (B) Labelled cells in the dorsal cochlear nucleus (DCN) as a result of labelling axons of the dorsal acoustic stria after unsuccessful injections, in this case caudal and ventral to facial gena (different animal than shown in A). (C) Retrogradely labelled cells in the ventral nucleus of the trapezoid body (VNTB) and (D) the rostral periolivary nucleus (RPO) nucleus. (E) Absence of labelled cells in the nucleus of lateral superior olive (LSO) following injections of the bidirectional tracer biocytin at the centre of the floor of the fourth ventricle. (F and G) High magnification images of medial olivocochlear (MOC) neurons with their extensive dendrites in (F) the VNTB and (G) the RPO. (H) MOC axons (arrows) ascending from their cell bodies in the superior olivary complex and coursing dorso-medially to the surface of the brainstem just beneath the floor of the fourth ventricle. Scale bars in A-E = 300µm, in F-H = 160µm.
Figure 3-3: Photomicrographs showing the results of successful (A and C-G) and unsuccessful (B) injections in mouse. (A) An example of a successful injection of the bidirectional tracer biocytin at the centre of the floor of the fourth ventricle. (B) Labelled cells in the dorsal cochlear nucleus (DCN) as a result of labelling axons of the dorsal acoustic stria after unsuccessful injections, in this case caudal and ventral to facial genua (different animal than shown in A). (C) Retrogradely labelled cells in the ventral nucleus of the trapezoid body (VNTB) and (D) the rostral periolivary (RPO) nucleus. (E and F) High magnification images of medial olivocochlear (MOC) neurons with their extensive dendrites in (E) the VNTB and (F) the RPO nucleus. (G) MOC axons (arrows) ascending from their cell bodies in the superior olivary complex and coursing dorsomedially to the surface of the brainstem just beneath the floor of the fourth ventricle. Scale bars in A-D = 100 µm, in E-G = 50 µm.
In animals without evidence of labelled cells in the DCN (Figures 3.4A-C), biocytin injections resulted in retrograde labelling of cells in the SOC nuclei. In guinea pigs, these labelled cells were distributed bilaterally throughout the medio-lateral and rostro-caudal regions of the VNTB, RPO and DMPO nuclei, while in rats and mice these labelled cells were only found in the VNTB and RPO nuclei (Figures 3.1C-E, 3.2C-D and 3.3C-D). The retrogradely labelled cells in the SOC nuclei in guinea pigs, rats and mice have been previously described as the place where MOC neurons are located (White and Warr, 1983, Robertson, 1985, Robertson et al., 1987a, Robertson et al., 1987c, Aschoff and Ostwald, 1988, Campbell and Henson, 1988, Vetter and Mugnaini, 1992). In all species, there were no biocytin-labelled cells evident within or around the margin of the LSO nucleus suggesting the LOC neurons were not labelled (Figures 3.1F, 3.2E and 3.3C).

Figure 3-4: Photomicrographs showing the absence of retrogradely labelled cells in the dorsal cochlear nucleus (DCN) following successful injections of the tracer biocytin at the centre of the floor of the fourth ventricle in (A) the guinea pigs, (B) rats and (C) the mouse. Scale bars in A-C = 100µm.
The cell bodies of the labelled MOC neurons were filled with a dense reaction product that commonly spread to the primary, secondary and even more distal dendritic branches. In guinea pigs, the cell body areas of labelled cells in the VNTB ranged from 139-298µm² (mean = 213.5, SD = 37.4µm², n = 38), in the RPO ranged from 107-329µm² (mean = 206.5, SD = 47.6µm², n = 222) and in the DMPO ranged from 119-291µm² (mean = 194.3, SD = 35.4µm², n = 45). The average diameter of labelled cells was 21.4µm (SD = 2.9µm) in the VNTB, 20.6µm (SD = 3.1µm) in the RPO and 21.1µm (SD = 2.4µm) in the DMPO. In rats, the cell body area ranged from 111-320µm² for the VNTB cells (mean = 189.9, SD = 47.7µm², n = 450) and 119-217µm² for the RPO cells (mean = 128.6, SD = 18.7µm², n = 347). The average diameter of labelled cells was 17.1µm (SD = 4µm) in the VNTB and 15.3µm (SD = 2.9µm) in the RPO. In the mouse, the cell body area ranged from 91-231µm² for the VNTB cells (mean = 147.1, SD = 27.6µm², n = 129) and 79-213µm² for the RPO cells (mean = 144.8, SD = 25.8µm², n = 129). The average diameter of the labelled cells was 15.7µm (SD = 2.5µm) in the VNTB and 15.2µm (SD = 2.5µm) in the RPO.

Retrogradely labelled cells in the VNTB, RPO and DMPO typically had several dendrites which radiated in different directions from the cell body. Based on their morphological features, these cells were categorized as multipolar cells which is consistent with previous findings in guinea pigs, rats and mice (Figures 3.1G-I, 3.2F-G and 3.3E-F) (White and Warr, 1983, Robertson, 1985, Aschoff and Ostwald, 1987, Robertson et al., 1987a, Robertson et al., 1987c, Campbell and Henson, 1988, Horváth et al., 2000).

The number of retrogradely labelled cells was counted bilaterally in the VNTB, RPO and DMPO following biocytin injection (Tables 3.1, 3.2 and 3.3 and Figures 3.5 and 3.6). The numbers provided in the tables and figures correspond to the total number of labelled cells on both sides of the brainstem. In two rats (R-2014-14 and R-2014-16), only 4/7 vials were used and stained for biocytin injections. Therefore, in these animals the estimated number of retrogradely labelled cells was mathematically corrected as follows: total number of cells = (total number of cell counted * total number of vials)/ number of vials used. In all species, labelled cells in the RPO were often more numerous than those in the VNTB and DMPO. In one rat (R-2014-40), only a few cells in the VNTB were labelled, most likely because the injection of the neuronal tracer at the floor of the fourth ventricle was more rostral and superficial. In the remaining animals, many cells were labelled in the nuclei of origin of MOC
neurons. The total number of labelled cells in guinea pigs ranged from 93-424 and in rats ranged from 13-1241. In the mouse, the total number of labelled cells was 432. These findings indicate that many MOC axons were labelled following biocytin injection.

Table 3-1

Numbers of biocytin-labelled medial olivocochlear (MOC) neurons in the ventral nucleus of the trapezoid body (VNTB), the rostral periolivary nucleus (RPO) and the dorsomedial periolivary nucleus (DMPO) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>Labelled cells in the VNTB</th>
<th>Labelled cells in the RPO</th>
<th>Labelled cells in the DMPO</th>
<th>Total of labelled MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-2017-76</td>
<td>237 (55.9%)</td>
<td>99 (23.3%)</td>
<td>88 (20.8%)</td>
<td>424</td>
</tr>
<tr>
<td>GP-2016-73</td>
<td>62 (17.1%)</td>
<td>292 (80.7%)</td>
<td>8 (2.2%)</td>
<td>362</td>
</tr>
<tr>
<td>GP-2014-42</td>
<td>19 (5.4%)</td>
<td>324 (92.3%)</td>
<td>8 (2.3%)</td>
<td>351</td>
</tr>
<tr>
<td>GP-2013-11</td>
<td>148 (46.2%)</td>
<td>158 (49.4%)</td>
<td>14 (4.4%)</td>
<td>320</td>
</tr>
<tr>
<td>GP-2014-41</td>
<td>16 (5%)</td>
<td>276 (87.1%)</td>
<td>25 (7.9%)</td>
<td>317</td>
</tr>
<tr>
<td>GP-2017-78</td>
<td>18 (6.8%)</td>
<td>245 (92.1%)</td>
<td>3 (1.1%)</td>
<td>266</td>
</tr>
<tr>
<td>GP-2017-75</td>
<td>11 (4.6%)</td>
<td>226 (95.4%)</td>
<td>0 (0%)</td>
<td>237</td>
</tr>
<tr>
<td>GP-2015-BBE</td>
<td>0 (0%)</td>
<td>160 (100%)</td>
<td>0 (0%)</td>
<td>160</td>
</tr>
<tr>
<td>GP-2015-ID298</td>
<td>13 (13%)</td>
<td>74 (74%)</td>
<td>13 (13%)</td>
<td>100</td>
</tr>
<tr>
<td>GP-2016-71</td>
<td>50 (53.8%)</td>
<td>28 (30.1%)</td>
<td>15 (16.1%)</td>
<td>93</td>
</tr>
</tbody>
</table>
Table 3-2
Numbers of biocytin-labelled medial olivocochlear (MOC) neurons in the ventral nucleus of the trapezoid body (VNTB) and the rostral periolivary nucleus (RPO) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats

<table>
<thead>
<tr>
<th>Experiment in rats</th>
<th>Labelled cells in the VNTB</th>
<th>Labelled cells in the RPO</th>
<th>Total of MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2014-16</td>
<td>338 (27.2%)</td>
<td>903 (72.8%)</td>
<td>1241</td>
</tr>
<tr>
<td>R-2014-14</td>
<td>302 (47.5%)</td>
<td>334 (52.5%)</td>
<td>636</td>
</tr>
<tr>
<td>R-2014-29</td>
<td>245 (60.9%)</td>
<td>157 (39.1%)</td>
<td>402</td>
</tr>
<tr>
<td>R-2014-53</td>
<td>150 (44.4%)</td>
<td>188 (55.6%)</td>
<td>338</td>
</tr>
<tr>
<td>R-2014-37</td>
<td>105 (43.6%)</td>
<td>136 (56.4%)</td>
<td>241</td>
</tr>
<tr>
<td>R-2015-55</td>
<td>4 (2.1%)</td>
<td>185 (97.9%)</td>
<td>189</td>
</tr>
<tr>
<td>R-2014-32</td>
<td>108 (58.7%)</td>
<td>76 (41.3%)</td>
<td>184</td>
</tr>
<tr>
<td>R-2014-25</td>
<td>72 (39.4%)</td>
<td>111 (60.6%)</td>
<td>183</td>
</tr>
<tr>
<td>R-2014-28</td>
<td>60 (34.7%)</td>
<td>113 (65.3%)</td>
<td>173</td>
</tr>
<tr>
<td>R-2014-35</td>
<td>111 (72.5%)</td>
<td>42 (27.5%)</td>
<td>153</td>
</tr>
<tr>
<td>R-2015-63</td>
<td>7 (5.9%)</td>
<td>111 (94.1%)</td>
<td>118</td>
</tr>
<tr>
<td>R-2014-30</td>
<td>17 (16.2%)</td>
<td>88 (83.8%)</td>
<td>105</td>
</tr>
<tr>
<td>R-2015-64</td>
<td>66 (100%)</td>
<td>0 (0%)</td>
<td>66</td>
</tr>
<tr>
<td>R-2014-40</td>
<td>13 (100%)</td>
<td>0 (0%)</td>
<td>13</td>
</tr>
</tbody>
</table>
Figure 3-5: Counts of medial olivocochlear (MOC) neurons in the ventral nucleus of the trapezoid body (VNTB), the rostral periolivary nucleus (RPO) and the dorsomedial periolivary nucleus (DMPO) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs.

Figure 3-6: Counts of medial olivocochlear (MOC) neurons in the ventral nucleus of the trapezoid body (VNTB) and the rostral periolivary nucleus (RPO) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats.
Table 3-3
Numbers of biocytin-labelled medial olivocochlear (MOC) neurons in the ventral nucleus of the trapezoid body (VNTB) and the rostral periolivary nucleus (RPO) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the mouse

<table>
<thead>
<tr>
<th>Experiment in mouse</th>
<th>Labelled cells in the VNTB</th>
<th>Labelled cells in the RPO</th>
<th>Total of MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-2015-08</td>
<td>185 (42.8%)</td>
<td>247 (57.2%)</td>
<td>432</td>
</tr>
</tbody>
</table>

3.2.2. MOC Axons and their Collateral Branches in the Cochlear Nucleus

Following biocytin injection, axons of MOC neurons in all species could be traced in the sections. The axons were shown to emanate from the rostro-caudal extension of the VNTB, RPO and DMPO and travel dorso-medially to the surface of the brainstem just beneath the floor of the fourth ventricle at the level of the genua of the seventh nerve (Figures 3.2H-3.3G). The axons were then found to course laterally beneath the vestibular nuclei, follow the vestibular nerve root and leave the brainstem.

The axons of MOC neurons were also shown to leave the main efferent bundle at several points along the course of the bundle (Figures 3.7A, 3.8A and 3.9A) and innervate the medial edge of the VCN (Figures 3.7B, 3.8B and 3.9B). These collateral branches then ramified, entered the VCN and terminated in the core of the PVCN and AVCN (Figures 3.7C, 3.8C and 3.9C). In all species, there were slightly more collateral branches of MOC axons in the core of the AVCN than observed in the PVCN. Axonal projections of MOC neurons were also observed in the granule cell layer separating the dorsal from the ventral subdivisions of the CN (Figures 3.7D, 3.8D and 3.9D). These collateral branches were usually found at the medial extent of the granule cell layer. In all species, MOC axons gave off collateral branches to the vestibular nuclei and the DCN (Figures 3.7E-F, 3.8E-F and 3.9E-F). The locations of MOC collateral branches and their terminations in the DCN and VCN in guinea pig (GP-2014-41), rat (R-2014-29) and mouse (M-2015-8) are shown in Figures 3.10, 3.11 and 3.12.
Figure 3-7: Photomicrographs showing the locations of labelled medial olivocochlear (MOC) collateral branches in the guinea pigs. (A) MOC axons send collateral branches to the ventral cochlear nucleus (VCN). High magnification images of MOC collateral branches in (B) the medial edge of the VCN, (C) the core of the VCN, (D) the granule cell layer (GCL), (E) the vestibular nucleus and (F) the dorsal cochlear nucleus (DCN), as indicated by arrows. Scale bars in A-F = 50µm.
Figure 3-8: Photomicrographs showing the locations of labelled medial olivocochlear (MOC) collateral branches in the rats. (A) MOC axons send collateral branches to the ventral cochlear nucleus (VCN). High magnification images of MOC collateral branches in (B) the medial edge of the VCN, (C) the core of the VCN, (D) the granule cell layer (GCL), (E) the vestibular nucleus and (F) the dorsal cochlear nucleus (DCN), as indicated by arrows. Scale bars in A-F = 100µm.
Figure 3-9: Photomicrographs showing the locations of labelled medial olivocochlear (MOC) collateral branches in the mouse. (A) MOC axons send collateral branches to the ventral cochlear nucleus (VCN). High magnification images of MOC collateral branches in (B) the medial edge of the VCN, (C) the core of the VCN, (D) the granule cell layer (GCL), (E) the vestibular nucleus and (F) the dorsal cochlear nucleus (DCN), as indicated by arrows. Scale bars in A-F = 50µm.
Figure 3-10: (A-J) A series of drawings of transverse (60μm) sections of the dorsal (DCN) and ventral cochlear nucleus (VCN) (distance between sections approximately 420μm) demonstrating the locations of medial olivocochlear (MOC) collateral branches in the granule cell layer (GCL), the DCN and medial edge and core of the VCN following biocytin injections at the centre of the floor of the fourth ventricle in the guinea pigs. A-J: caudal-rostral. Scale bar = 500μm.
Figure 3-11: (A-F) A series of drawings of transverse (60µm) sections of the dorsal (DCN) and ventral cochlear nucleus (VCN) (distance between sections approximately 420µm) demonstrating the locations of medial olivocochlear (MOC) collateral branches in the granule cell layer (GCL), the DCN, and medial edge and core of the VCN following the bidirectional tracer biocytin injections at the centre of the floor of the fourth ventricle in the rats. A-F: caudal-rostral. Scale bar = 1000µm.
Figure 3-12: (A-F) A series of drawings of transverse (40µm) sections of the dorsal (DCN) and ventral cochlear nucleus (VCN) (distance between sections approximately 280µm) demonstrating the locations of medial olivocochlear (MOC) collateral branches in the granule cell layer (GCL), the DCN, and medial edge and core of the VCN following the bidirectional tracer biocytin injections at the centre of the floor of the fourth ventricle in the mouse. A-F: caudal-rostral. Scale bar = 500µm.
Collateral branches of MOC axons in the VCN split into two or three additional branches, and formed numerous enlargements along or at the tips of MOC collateral branches that we called *en passant* and terminal varicosities (Figure 3.13A-C). Varicosities in the VCN were mostly round in shape and stained darkly and uniformly which made them easy to identify. Tables 3.4, 3.5 and 3.6 and Figures 3.14 and 3.15 show the number of MOC *en passant* and terminal varicosities in the VCN of the guinea pigs, rats and the mouse. The numbers provided in the tables and figures correspond to the total number of MOC *en passant* and terminal varicosities in the VCN on both sides of the brainstem.

Figure 3-13: High magnification images of medial olivocochlear (MOC) *en passant* and terminal varicosities in the ventral cochlear nucleus (VCN) following successful injections at the centre of the floor of the fourth ventricle in (A) the guinea pigs, (B) rats and (C) the mouse. Scale bars in A = 200µm and in B and C = 50µm.
Table 3-4
Numbers of medial olivocochlear (MOC) *en passant* and terminal varicosities in the ventral cochlear nucleus (VCN) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs. Numbers in brackets is percentage

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>En passant and terminal swellings of MOC collateral branches in different regions of the VCN</th>
<th>Total of <em>en passant</em> and terminal swellings of MOC neurons in different regions of the VCN</th>
<th>Swellings/MOC neurons ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Core of the VCN</td>
<td>Medial edge of the VCN</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GP-2017-76</td>
<td>3161 (47.5%)</td>
<td>1870 (28.1%)</td>
<td>1628 (24.4%)</td>
</tr>
<tr>
<td>GP-2016-73</td>
<td>1680 (25.4%)</td>
<td>3088 (46.7%)</td>
<td>1845 (27.9%)</td>
</tr>
<tr>
<td>GP-2014-41</td>
<td>1958 (41.7%)</td>
<td>1389 (29.6%)</td>
<td>1350 (28.7%)</td>
</tr>
<tr>
<td>GP-2014-42</td>
<td>1242 (27.6%)</td>
<td>1405 (31.2%)</td>
<td>1852 (41.2%)</td>
</tr>
<tr>
<td>GP-2013-11</td>
<td>1378 (33.3%)</td>
<td>1541 (37.2%)</td>
<td>1222 (29.5%)</td>
</tr>
<tr>
<td>GP-2017-78</td>
<td>2060 (51.4%)</td>
<td>904 (22.5%)</td>
<td>1048 (26.1%)</td>
</tr>
<tr>
<td>GP-2017-75</td>
<td>1317 (35.3%)</td>
<td>1224 (32.8%)</td>
<td>1190 (31.9%)</td>
</tr>
<tr>
<td>GP-2015-BBE</td>
<td>876 (34.9%)</td>
<td>1086 (43.3%)</td>
<td>546 (21.8%)</td>
</tr>
<tr>
<td>GP-2015-ID298</td>
<td>882 (36.1%)</td>
<td>506 (20.8%)</td>
<td>1053 (43.1%)</td>
</tr>
<tr>
<td>GP-2016-71</td>
<td>426 (18.6%)</td>
<td>518 (22.6%)</td>
<td>1351 (58.8%)</td>
</tr>
<tr>
<td>Experiment in rats</td>
<td>En passant and terminal swellings of MOC collateral branches in different regions of the VCN</td>
<td>Total of en passant and terminal swellings of MOC collateral branches in the VCN</td>
<td>Swellings /MOC neurons ratio</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td></td>
<td>Core of the VCN</td>
<td>Medial edge of the VCN</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>R-2014-16</td>
<td>247 (4.6%)</td>
<td>5032 (93%)</td>
<td>131 (2.4%)</td>
</tr>
<tr>
<td>R-2014-53</td>
<td>201 (6.7%)</td>
<td>2174 (73%)</td>
<td>604 (20.3%)</td>
</tr>
<tr>
<td>R-2014-37</td>
<td>798 (27.3%)</td>
<td>1301 (44.4%)</td>
<td>828 (28.3%)</td>
</tr>
<tr>
<td>R-2014-29</td>
<td>391 (13.5%)</td>
<td>1948 (67.5%)</td>
<td>547 (19%)</td>
</tr>
<tr>
<td>R-2014-14</td>
<td>307 (12.6%)</td>
<td>2056 (84%)</td>
<td>83 (3.4%)</td>
</tr>
<tr>
<td>R-2015-55</td>
<td>65 (3.7%)</td>
<td>1568 (89.7%)</td>
<td>115 (6.6%)</td>
</tr>
<tr>
<td>R-2014-25</td>
<td>193 (14.4%)</td>
<td>938 (70.1%)</td>
<td>207 (15.5%)</td>
</tr>
<tr>
<td>R-2014-32</td>
<td>192 (15.5%)</td>
<td>831 (67%)</td>
<td>217 (17.5%)</td>
</tr>
<tr>
<td>R-2014-30</td>
<td>309 (34.4%)</td>
<td>361 (40.1%)</td>
<td>229 (25.5%)</td>
</tr>
<tr>
<td>R-2014-35</td>
<td>76 (8.7%)</td>
<td>655 (74.9%)</td>
<td>144 (16.4%)</td>
</tr>
<tr>
<td>R-2015-63</td>
<td>17 (2.4%)</td>
<td>684 (97.6%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>R-2015-64</td>
<td>0 (0%)</td>
<td>607 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>R-2014-28</td>
<td>16 (3.6%)</td>
<td>297 (66.9%)</td>
<td>131 (29.5%)</td>
</tr>
<tr>
<td>R-2014-40</td>
<td>0 (0%)</td>
<td>20 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

Table 3-5

Numbers of medial olivocochlear (MOC) en passant and terminal varicosities in the ventral cochlear nucleus (VCN) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats. Numbers in brackets is percentage.
Figure 3-14: Counts of medial olivocochlear (MOC) *en passant* and terminal varicosities in the ventral cochlear nucleus (VCN) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the guinea pigs.

Figure 3-15: Counts of medial olivocochlear (MOC) *en passant* and terminal varicosities in the ventral cochlear nucleus (VCN) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the rats.
Numbers of medial olivocochlear (MOC) en passant and terminal varicosities in the ventral cochlear nucleus (VCN) on both sides of the brainstem following successful injections at the centre of the floor of the fourth ventricle in the mouse. Numbers in brackets is percentage

<table>
<thead>
<tr>
<th>Experiment in mouse</th>
<th>En passant and terminal swellings of MOC collateral branches in different regions of the VCN</th>
<th>Total of en passant and terminal swellings of MOC collateral branches in the VCN</th>
<th>Swellings /MOC neurons ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core of the VCN</td>
<td>Medial edge of the VCN</td>
<td>Granule cell layer</td>
<td></td>
</tr>
<tr>
<td>M-2015-8</td>
<td>2523 (33.8%)</td>
<td>3590 (48.1%)</td>
<td>1349 (18.1%)</td>
</tr>
</tbody>
</table>

In four guinea pigs (GP-2017-76, GP-2014-41, GP-2017-78 and GP-2017-75), MOC en passant and terminal varicosities were observed primarily in the core of the VCN. In the remaining guinea pigs, however, most of the MOC axon varicosities were found in the granule cell layer (as shown in GP-2014-42, GP-2015-ID298 and GP-2016-71) or in the medial edge of the VCN (as shown in GP-2016-73, GP-2013-11 and GP-2015-BBE). In rats, the majority of MOC en passant and terminal varicosities were found at the medial edge of the VCN and to a lesser extent in the granule cell layer and the core of the PVCN and AVCN. In the mouse, the highest number of MOC en passant and terminal varicosities were observed in the medial edge of the VCN.

In all species, MOC en passant and terminal varicosities in the PVCN and AVCN were easy to distinguish because their diameters were almost twice the diameters of the parent MOC collateral branches. The average diameter of the MOC en passant and terminal varicosities was 2.49μm in guinea pigs (SD = 0.38μm, n = 58), 2.58μm in rats (SD = 0.33μm, n = 44) and 1.75μm in the mouse (SD = 0.77μm, n = 53). In addition, the average diameter of the MOC collateral branches was 1.5μm in guinea pigs (SD = 0.26μm, n = 58), 1.6μm in rats (SD = 0.16μm, n = 44) and 0.7μm in the mouse (SD = 0.16μm, n = 53). In both the guinea pigs and rats, en passant and terminal varicosities were 60% greater in diameter than the MOC collateral branch diameters in the VCN. In the mouse, they were 40% greater in diameter than the MOC collateral branches. The ratio between the number of anterogradely labelled en passant and terminal varicosities in the VCN and the number of retrogradely labelled cells in the nuclei of origin of MOC neurons varied from 12.8-24.7 in the guinea pig, 1.5-12.1 in the rat and 17.3 in the mouse. There was an apparent relationship between the total
number of the labelled cells in the VNTB, RPO and DMPO and the total number of labelled
MOC axonal varicosities in the VCN (Figure 3.16).

Figure 3-16: An apparent relationship exists between the total number of the biocytin-labelled medial
olivocochlear (MOC) neurons and the total number of MOC en passant and terminal varicosities in the
ventral cochlear nucleus on both sides of the brainstem in the guinea pigs and rats.

3.3. Discussion

This study shows that injections of biocytin at the centre of the floor of the fourth ventricle
can be used to label MOC neurons and their axons and collateral branches in the CN of
guinea pigs, rats and mice. Animals with retrograde labelling in any subdivision of the CN
were excluded from the analysis as retrogradely labelled cells in the CN may have local
axonal branches, and may give misleading results because they may originate from cells in
the CN, and not from the axonal branches of MOC neurons.

The locations of MOC neurons observed in this study are in broad agreement with other
studies of guinea pigs, rats and mice after tracer injection into the cochlea (White and Warr,
1983, Robertson et al., 1987a, Robertson et al., 1987c, Campbell and Henson, 1988, Winter
et al., 1989, Horváth et al., 2000). The large number of dendrites observed on neurons in the
known nuclei of origin of MOC neurons are also consistent with those previously described
using other methods (Adams, 1983a, White and Warr, 1983, Osen et al., 1984, Robertson et
The absence of labelled cells in the dorsal and ventral subdivisions of the CN and in the SOC regions (the LSO and MSO) in combination with the location of retrogradely labelled cells in the VNTB, RPO and DMPO confirm the injections were correctly placed in this study. Incorrect placement of the injection (more caudal and ventral to the facial genua) resulted in labelling of axons of the dorsal or/and intermediated acoustic stria, and hence labelled the fusiform and giant cells in the DCN or/and the octopus and multipolar cells in the VCN (Harrison and Irving, 1966a, Fernandez and Karapas, 1967, Adams and Warr, 1976, Adams, 1979, Smith and Rhode, 1989, Huffman and Covey, 1995).

In the present study, no LOC neurons were retrogradely labelled in the guinea pigs, rats and mouse. This might be because the LOC axon tract runs more lateral to the midline of the brainstem than where the biocytin injection was placed. It might also be because the axons of LOC neurons are thin and unmyelinated and biocytin is transported more readily by thick and myelinated axons (Warr and Guinan, 1979, Liberman, 1980, Guinan et al., 1983, White and Warr, 1983, Ginzberg and Morest, 1984, Liberman and Brown, 1986).

The location of the axonal branches of MOC neurons in the VCN and vestibular nuclei labelled in this study are comparable to those described previously in cats (Brown et al., 1988) and other studies of mice (Brown et al., 1991, Brown, 1993, Brown and Levine, 2008), rats (White and Warr, 1983, Horváth et al., 2000) and guinea pigs (Winter et al., 1989). MOC collateral branches to the VCN in all species have been shown to innervate the medial edge of the VCN, the granule cell layer and the core of the VCN. In this study, there was no labelling of cells in the LSO nucleus, suggesting that collateral branches observed in the VCN in guinea pigs, rats and mice arise solely from MOC axons (Guinan et al., 1983, White and Warr, 1983, Robertson, 1985, Brown, 1987, Campbell and Henson, 1988, Vetter and Mugnaini, 1992).

The present study also shows that successful location of the injection at the centre of the floor of the fourth ventricle leads to labelling of axonal branches in all layers of the DCN, but no retrograde labelling of cells in the DCN. These branches might be due to inadvertent
labelling of somatosensory pathways, as the DCN receives projections from some nuclei of the somatosensory brainstem structures, including the dorsal column and spinal trigeminal nuclei (Itoh et al., 1987, Weinberg and Rustioni, 1987, Haenggeli et al., 2005). In cats, Itoh et al. (1987) found after injecting a neuronal tracer (wheat germ agglutinated horseradish peroxidase) into the dorsal column and the spinal trigeminal nuclei that both nuclei of the dorsal column and the spinal trigeminal send axons directly to the pyramidal cell layer of the DCN and the granule cell layer of the VCN. In rats, guinea pigs and hedgehogs, these brainstem somatosensory projections have been shown to terminate in the deep cell layer of the DCN and the granule cell layer of the PVCN and AVCN (Wright and Ryugo, 1996, Wolff and Künzle, 1997, Zhou and Shore, 2004, Haenggeli et al., 2005). However, the floor of the fourth ventricle is not near the dorsal column or spinal trigeminal nuclei, and incorrect location of the injection (more rostral and dorsal to the facial genua) did not result in labelling any of the axonal branches in the DCN or the VCN.

In rodents, Brown et al. (1988) studied the terminal distribution of the MOC axons in the CN. They injected the anterograde axonal transport of horseradish peroxidase into the cochlea and found that the axonal branches of MOC neurons terminated primarily in the VCN, but a few branches terminated in the deep cell layer of the DCN. Previous studies in several species, by staining brain sections using AChE, also found AChE-positive axonal branches mostly within the PVCN and AVCN, but some within the strial corner of the DCN and the superficial cell layer of the DCN (Martin, 1981, Osen et al., 1984, Brown et al., 1988). Therefore, it seems likely that the axonal branches in the DCN in the present study originated from the MOC axons.

A previous study in guinea pigs found that the total number of labelled MOC neurons following bilateral cochlear injections ranged from 643-1948 (Robertson et al., 1987b). Other studies in rats found that the sum of labelled MOC neurons following unilateral cochlear applications ranged from 189-320, which presumably represents half of the total number of MOC neurons (White and Warr, 1983, Vetter and Mugnaini, 1992). In mice, Campbell and Henson (1988) also found that the total number of the retrogradely labelled cells in the nuclei of origin of MOC neurons following unilateral cochlear injections was 164, which also represents half of the total number of MOC neurons. Indeed, injections of retrograde axonal tracer into the intraganglionic spiral bundle of the cochlea employed in
the previous studies labelled the axons of crossed and uncrossed MOC neurons and their collateral branches in the CN. In the present study, the total number of labelled MOC neurons ranged from 93-424 in guinea pigs, from 13-1241 in rats and was 432 in the mouse, which presumably represents the axons of crossed MOC neurons. In comparison to the estimated numbers made by others, our injections could result in labelling a large proportion of MOC neurons (specifically the crossed MOC axons) in the rats and mouse. In guinea pigs, the total number of labelled MOC neurons was considerably lesser than those observed in the study of Robertson and colleagues (1987b), indicating that our injections only label a small part of the crossed MOC system population.

In summary, biocytin injections at the centre of the floor of the fourth ventricle where MOC axons are grouped in a tight bundle can be used to label MOC neurons and their collateral branches in the VCN of guinea pigs, rats and mice. This was confirmed by the location and morphology of biocytin-labelled cells in the SOC nuclei, the dorso-medial trajectory of their axons projecting to the surface of the floor of the fourth ventricle and the appearance of a separate well-labelled bundle at the point of exit of the OCB from the brainstem. Collateral branches of MOC neurons formed en passant and terminal varicosities in the VCN. These varicosities terminated predominately in the medial edge of the VCN, the granule cell layer and the core of the PVCN and AVCN.
Chapter 4

Anatomical Relationship between Multipolar (T-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat

4.1. Introduction

The previous experiments showed that injections of the neuronal tracer biocytin at the centre of the floor of the fourth ventricle were able to label axonal varicosities of MOC neurons in all subdivisions of the CN (see Chapter 3). Therefore, the main objective of the present study was to label multipolar (T-stellate) cells in the VCN of guinea pigs and rats by injection of the retrograde tracer FG into the CNIC and to investigate whether these cells receive synaptic innervation from MOC axonal varicosities. For details of the methods see Chapter 2.

4.2. Results

4.2.1. Locations and Characteristics of FG Injection Sites

Iontophoretic injections of FG into the CNIC were successfully performed in 13 guinea pigs and 25 rats. In each animal, two FG injections were made into the CNIC at various depths in an attempt to label a greater number of cells than might be obtained with a single injection. The two injections into the CNIC were separated in depth by 0.55mm in the guinea pigs and 0.25mm in the rats. Light microscopy showed that in almost all animals the two injection sites into the CNIC were not easily distinguishable from each other, as they commonly fused together, covering most of the CNIC. These injections resulted in large and roughly spherical injection sites. The injection sites were commonly characterized by a small necrotic core at the centre, surrounded by an area of densely FG labelled cell bodies and a peripheral halo of lightly labelled neuropil (Figures 4.1A and 4.2A). In all successfully labelled animals, retrograde tracer injected in the CNIC did not encroach into any additional structures, including the contralateral IC, the LL, the SOC or the superior colliculus. Injections into the CNIC were usually located in the central region of the CNIC, but were sometimes located in the ventral or dorsal regions of the CNIC. The different injection sites into the CNIC are discussed in Section 4.2.2.
Figure 4-1: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the inferior colliculus (IC) in guinea pigs. (A) The FG injection site in the IC. (B) FG labelled cells in the contralateral IC. (C and D) FG labelled cells in the ipsilateral dorsal (DNLL), intermediate (INLL) and ventral (VNLL) nuclei of the lateral lemniscus. (E, F and G) FG labelled cells in the ipsilateral lateral superior olive (LSO), the medial superior olive (MSO) and the dorsomedial periolivary nucleus (DMPO). Scale bars in A = 250µm, in B-G = 200µm.
Figure 4-2: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the inferior colliculus (IC) in rats. (A) The FG injection site in the IC. (B) FG labelled cells in the contralateral IC. (C and D) FG labelled cells in the ipsilateral dorsal (DNLL), intermediate (INLL) and ventral (VNLL) nuclei of the lateral lemniscus. (E and F) FG labelled cells in the ipsilateral lateral superior olive (LSO), the medial superior olive (MSO) and the dorsomedial periolivary nucleus (DMPO). Scale bars in A = 250µm, in B-F = 200µm.

In the 13 successfully labelled guinea pigs, the diameter of the FG injection sites varied from 384-1257µm (mean = 1002.4, SD = 206.6µm) in the medio-lateral direction, from 670-1123µm (mean = 893.9, SD = 121.6µm) in the dorso-ventral direction and from 720-1620µm (mean = 1186.1, SD = 258.2µm) in the rostro-caudal direction. In the 25 successfully labelled rats, the diameter of the injection sites varied from 327-988µm (mean = 593, SD = 207.7µm) in the medio-lateral direction, from 422-1290µm (mean = 745.2, SD = 312.2µm) in the dorso-ventral direction and from 840-1320µm (mean = 1065.9, SD = 138.4µm) in the rostro-caudal direction.
4.2.2. Distribution of Labelled cells in the Nuclei of the Auditory System

The FG injections labelled cells in a number of nuclei of the auditory system. The results were similar in the guinea pigs and rats and so are described together. Following successful injections in the CNIC, labelled cells were found in the contralateral IC, the dorsal, intermediate and ventral nuclei of the LL and some nuclei of the SOC, including the LSO, MSO and DMPO. Labelled cells were also observed in the dorsal and ventral subdivisions of the CN. No labelled cells were observed in the MNTB, superior colliculus, trigeminal nuclei, or dorsal column region.

The pattern of retrograde labelling in the nuclei of the IC, LL and SOC in the guinea pigs and rats is shown in Figures 4.1 and 4.2. The general labelling pattern within these auditory structures will be briefly described first and then the labelling in the CN is more extensively described, particularly the VCN, since this region was the main focus of this study. A substantial number of small and large multipolar cells were labelled in the contralateral CNIC (Figures 4.1B and 4.2B). Bilateral labelling of the LL appeared in the DNLL, with more prominent labelling contralateral to the injection site. Labelled cells were also observed in the ipsilateral, but not the contralateral, intermediate nucleus of the lateral lemniscus (INLL) and VNLL (Figures 4.1C-D and 4.2C-D). The retrogradely labelled cells in the DNLL, INLL and VNLL had elongated, ovoid, multipolar or round cell bodies. Projections from the nuclei of the LL to the CNIC described in this study are consistent with previous similar studies in other species (Beyerl, 1978, Adams, 1979, Coleman and Clerici, 1987, Kelly et al., 1998, Ito et al., 2008, Kelly et al., 2009, Hatano et al., 2012). In addition to labelling in all three subdivisions of the LL, numerous FG labelled cells were seen in the SOC regions (Figures 4.1E-G and 4.2E-F). Multipolar and bipolar cells were observed bilaterally in the LSO. On the side ipsilateral to the FG injection, the MSO contained bipolar cells whereas the DMPO included multipolar cells, as previously described in guinea pigs (Schofield and Cant, 1992) and rats (Beyerl, 1978, Coleman and Clerici, 1987, Kelly et al., 1998, Schaeffer et al., 2003, Ito et al., 2008, Fredrich et al., 2009, Kelly et al., 2009).

In the CN, many cells were labelled in the contralateral DCN, PVCN and AVCN with relatively few labelled cells ipsilateral to the injection site. The general distribution of FG positive cells within the contralateral DCN, PVCN and AVCN in the guinea pigs and rats is shown in Figures
4.3 and 4.4. The distribution of FG labelled cells in each subdivision of the CN was fairly similar in the guinea pigs and rats following injections into the central regions of the CNIC (Figure 4.5). However, in three rats (R-2015-56, 57 and 60) the injection was into the ventral region of the CNIC and resulted in labelling of cells in the dorsal regions of the contralateral DCN and VCN (Figure 4.6). In another rat (R-2014-14), the FG injections were into the dorsal region of the CNIC and resulted in labelling of cells in the ventral regions of the DCN and VCN, on the side contralateral to the FG injection (Figure 4.7). These topographic projection results are in agreement with results from other studies (Beyerl, 1978, Adams, 1979, Ryugo et al., 1981, Coleman and Clerici, 1987, Oliver, 1987).
Figure 4-3: Photomicrographs of retrograde labelling in the cochlear nucleus (CN) after a unilateral injection of FG into the inferior colliculus (IC) in guinea pigs. (A) FG labelled cells in the contralateral dorsal DCN. (B) High magnification image of FG labelled fusiform (pyramidal) cells in the DCN. (C and D) FG labelled cells in the posterior (PVCN) and anterior (AVCN) ventral cochlear nuclei. (E and F) High magnification images of FG labelled multipolar cells with their extensive dendrites in the VCN contralateral to the FG injection site. Scale bars in A, C and D = 250µm, in B, E and F = 50µm.
Figure 4-4: Photomicrographs of retrograde labelling in the cochlear nucleus (CN) after a unilateral injection of FG into the inferior colliculus (IC) in rats. (A) FG labelled cells in the contralateral dorsal DCN. (B) High magnification image of FG labelled fusiform (pyramidal) cells in the DCN. (C and D) FG labelled cells in the contralateral posterior (PVCN) and anterior (AVCN) ventral cochlear nuclei. (E, F and G) High magnification images of FG labelled multipolar cells with their extensive dendrites in the VCN contralateral to the FG injection site. Scale bars in A, C and D = 100µm, in B, E, F and G = 50µm.

In the DCN, labelled cells were generally located within the inner layers (layer 2 and layer 3) in agreement with previous reports (Adams, 1979, Brunso-Bechtold et al., 1981, Webster and Trune, 1982, Oliver, 1984, Hackney et al., 1990, Hatano et al., 2012). Fusiform cells were the most numerous labelled cells in the DCN (Figures 4.3B and 4.4B). Giant cells and other cells of varying shape were also labelled within the deeper layers of the DCN consistent with
results obtained by Schofield and Cant (1996b) in guinea pigs and by Coleman and Clerici (1987), Beyerl (1978), Kelly et al. (2009) and Hatano et al. (2012) in rats. The retrograde labelled cells in the DCN often gave rise to three or four dendrites that left the cell bodies at many different angles. The apical dendrites were directed toward the external surface of the DCN, whereas the basal dendritic field extended to the inner layers (layer 2 and layer 3) of the DCN.

Labelled cells that were located throughout much of the VCN could be characterized as multipolar cells. These observations are compatible with earlier findings in guinea pigs (Pirsi, 1968, Hackney, 1987, Schofield and Cant, 1996b) and rats (Beyerl, 1978, Coleman and Clerici, 1987, Kelly et al., 2009, Hatano et al., 2012). Figures 4.3E-F and 4.4E-G show examples of multipolar labelled cells in the contralateral PVCN and AVCN in guinea pigs and rats. In some cases, FG labelled cells in the PVCN and AVCN had polygonal cell bodies which gave rise to three or more dendrites that extended in multiple directions. In other cases, the cell bodies were rounded and gave rise to three, four and sometimes five, dendrites. Multipolar cells possessing an elongated cell body were also observed along the margins of both the PVCN and AVCN. These cells commonly had three primary dendrites. Labelled cells with polygonal cell bodies in the PVCN and AVCN were labelled in greater number in comparison to others. Neither octopus cells nor globular bushy cells in the PVCN were labelled following the FG injections. In agreement with other studies, spherical bushy cells in the AVCN were also not labelled (Harrison and Warr, 1962, Osen, 1969, Beyerl, 1978, Webster and Trune, 1982, Hackney et al., 1990, Schofield and Cant, 1996b).
Figure 4-5: (A-H) A series of drawings of transverse (60µm) sections of the contralateral dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 420µm) showing the locations of labelled cells following the retrograde tracer FG injections into the central region of the inferior colliculus in guinea pig (R-2015-43). Section A is most caudal; H is most rostral. Scale bar = 500µm.

Figure 4-6: (A-G) A series of drawings of transverse (60µm) sections of the contralateral dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 420µm) showing the locations of labelled cells following the retrograde tracer FG injections into the ventral region of the inferior colliculus in rat (R-2015-57). Section A is most caudal; G is most rostral. Scale bar = 500µm.
Figure 4.7: (A-G) A series of drawings of transverse (60µm) sections of the contralateral dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 420µm) showing the locations of labelled cells following the retrograde tracer FG injections into the dorsal region of the inferior colliculus in rat (R-2014-14). Section A is most caudal; G is most rostral. Scale bar = 500µm.

In guinea pigs, the soma areas of the FG labelled multipolar cells that projected from the VCN to the CNIC ranged from 143-572µm² (mean = 302, SD = 87.5µm², n = 256) and the long axis of these cells ranged from 14-34µm (mean = 22.9, SD = 4.3µm). In rats, the cell body areas of the labelled multipolar cells in the VCN ranged from 97-266µm² (mean = 158.5, SD = 31.2µm², n = 258) and the long axis of these cells ranged from 10-25µm (mean = 16.2, SD = 2.5µm).

4.2.3. MOC Axonal Varicosities and Multipolar Cells in the Ventral Cochlear Nucleus

Combined anterograde and retrograde labelling experiments were successful in labelling both the CNIC and the floor of the fourth ventricle in 4 guinea pigs and 5 rats. Tables 4.1 and 4.2 summarise the number of FG labelled multipolar cells and biocytin-labelled MOC axonal varicosities in the VCN following successful injections in these guinea pigs and rats. In two rats (R-2014-14 and R-2014-16), only 4/7 vials were used and stained for FG injections. Therefore, in these animals the estimated number of FG labelled cells was mathematically corrected as follows: total number of cells = (total number of cell counted * total number of vials)/ number of vials used. Putative synaptic contacts between the multipolar cells and MOC axonal varicosities in the VCN ipsilateral and contralateral to the FG injection site were
examined. The criteria used to identify false or possibly true synaptic contacts using the light microscope are described in Chapter 2 (Section 2.7.).

Table 4-1
Numbers of FG and biocytin-labelled cells and medial olivocochlear (MOC) axonal varicosity in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the four successfully labelled guinea pigs

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>Total of labelled multipolar cells in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC axonal varicosity in the VCN contralateral to the FG injection site</th>
<th>Total of labelled MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-2014-42</td>
<td>3729</td>
<td>2229</td>
<td>351</td>
</tr>
<tr>
<td>GP-2014-41</td>
<td>3518</td>
<td>2283</td>
<td>317</td>
</tr>
<tr>
<td>GP-2015-ID298</td>
<td>1510</td>
<td>1306</td>
<td>100</td>
</tr>
<tr>
<td>GP-2015-BBE</td>
<td>1097</td>
<td>1629</td>
<td>160</td>
</tr>
</tbody>
</table>

Table 4-2
Numbers of FG and biocytin-labelled cells and medial olivocochlear (MOC) axonal varicosity in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the five successfully labelled rats

<table>
<thead>
<tr>
<th>Experiment in rats</th>
<th>Total of labelled multipolar cells in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC axonal varicosity in the VCN contralateral to the FG injection site</th>
<th>Total of labelled MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2015-55</td>
<td>5357</td>
<td>1095</td>
<td>189</td>
</tr>
<tr>
<td>R-2015-63</td>
<td>4801</td>
<td>410</td>
<td>118</td>
</tr>
<tr>
<td>R-2014-16</td>
<td>4012</td>
<td>3196</td>
<td>1241</td>
</tr>
<tr>
<td>R-2015-64</td>
<td>3489</td>
<td>472</td>
<td>66</td>
</tr>
<tr>
<td>R-2014-14</td>
<td>2892</td>
<td>1714</td>
<td>636</td>
</tr>
</tbody>
</table>

Close contact between retrogradely labelled cells and MOC axonal varicosities were extremely rare. In the four successfully double-labelled guinea pigs, GP-2014-41 had nine (0.26%) multipolar cells, GP-2014-42 had seven (0.19%) multipolar cells, GP-2015-ID298 had three (0.20%) multipolar cells and GP-2015-BBE had two (0.18%) multipolar cells with putative synaptic contacts in the VCN contralateral to the FG injection site. In the five labelled rats, R-2014-16 had eight (0.20%) multipolar cells, R-2014-14 had four (0.14%) multipolar cells, R-2015-64 had two (0.06%) multipolar cells and R-2014-55 had one (0.02%) multipolar cell with putative synaptic contacts in the VCN contralateral to the FG injection site. Tables 4.3 and 4.4 and Figures 4.8 and 4.9 summarise the numbers and locations of putative synaptic contacts between the retrogradely labelled multipolar cells and
anterogradely labelled MOC axonal varicosities in the VCN contralateral to the FG injection site in guinea pigs and rats.

Table 4-3
Numbers and location of putative synaptic contacts in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the four successfully labelled guinea pigs

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>Putative synaptic contacts on cell body</th>
<th>Putative synaptic contacts on dendrite</th>
<th>Medial edge of the VCN</th>
<th>Core of the VCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-2014-41</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>GP-2014-42</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>GP-2015-ID298</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>GP-2015-BBE</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 4-4
Numbers and location of putative synaptic contacts in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the five successfully labelled rats

<table>
<thead>
<tr>
<th>Experiment in rats</th>
<th>Putative synaptic contacts on cell body</th>
<th>Putative synaptic contacts on dendrite</th>
<th>Medial edge of the VCN</th>
<th>Core of the VCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2014-16</td>
<td>6</td>
<td>2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>R-2014-14</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>R-2015-64</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-55</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>R-2015-63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4-8: Counts of FG and biocytin-labelled cells following successful injections into the inferior colliculus and at the floor of fourth ventricle in four guinea pigs. Numbers above the blue columns indicate the number of FG labelled multipolar cells with putative synaptic contacts from biocytin-labelled medial olivocochlear (MOC) axonal varicosities in the ventral cochlear nucleus contralateral to the FG injection site.

Figure 4-9: Counts of FG and biocytin-labelled cells following successful injections into the inferior colliculus and at the floor of fourth ventricle in five rats. Numbers above the blue columns indicate the number of FG labelled multipolar cells with putative synaptic contacts from biocytin-labelled medial olivocochlear (MOC) axonal varicosity in the ventral cochlear nucleus contralateral to the FG injection site.
Axonal varicosities were observed in close contact with somata and proximal dendrites of the retrogradely labelled cells, though the majority was observed on somata. In the guinea pigs, the MOC axonal varicosities made putative synaptic contact with 15 (71.4%) cell bodies and/or six dendrites (28.6%) of the multipolar cells. In the rats, the MOC axonal varicosities made synaptic contact with 13 (86.7%) cell bodies and/or two dendrites (13.7%) of multipolar cells. Examples of putative synaptic contacts between multipolar cells and MOC axonal varicosities in the VCN contralateral to the FG injection site are shown in Figure 4.10. In one rat (R-2015-63), no putative synaptic contacts between multipolar cells and MOC axonal varicosities were found.

![Figure 4-10](image)

**Figure 4-10:** Light microscopic image showing synaptic contacts between biocytin-labelled medial olivocochlear axonal varicosities and FG labelled multipolar (T-stellate) cells in the ventral cochlear nucleus of (A and B) guinea pigs and (C and D) rats. Scale bars in A-D = 50µm.
In guinea pigs, a higher number of putative synapses (14/21) was found in the core of the VCN, compared to the rats where most putative synapses (13/15) were found in the medial edge of the VCN, particularly in the PVCN. This could be because the MOC axonal varicosities in the most successfully labelled guinea pigs were located primarily in the core of the VCN, whereas in the rats they were located mainly in the medial edge of the PVCN and AVCN, as previously shown in Chapter 3 (Section 3.2.2.). In all successfully labelled animals, no synaptic contacts between multipolar cells and MOC axonal varicosities were observed in the VCN ipsilateral to the FG injection site. On both sides of the brainstem, however, many MOC axonal varicosities were observed to make possible contact with VCN structures that were not labelled with FG (Figure 4.11). For more details of these unlabelled structures in the VCN contralateral and ipsilateral to the FG injection site see Section 4.3 below.

![Figure 4-11: Photomicrographs showing examples of putative synaptic contacts between medial olivocochlear axonal varicosities (arrows) and unlabelled ventral cochlear nucleus structures (stars) in (A) guinea pigs and (B) rats. Scale bars in A-B = 40µm.](image)

**4.3. Discussion**

This study of guinea pigs and rats is consistent with previous studies that show that the IC receives inputs from several lower brainstem nuclei in the auditory pathway (Beyerl, 1978, Adams, 1979, Ryugo et al., 1981, Coleman and Clerici, 1987, Oliver, 1987, Schofield and Cant, 1996b, Kelly et al., 1998, Schaeffer et al., 2003, Ito et al., 2008, Fredrich et al., 2009, Kelly et al., 2009, Hatano et al., 2012). Our findings show that tracer injections into the CNIC resulted in retrograde labelling of cells in the contralateral IC, ipsilaterally in nuclei of the LL.
and some SOC nuclei, including the LSO, MSO and DMPO. Many labelled cells were also observed in the contralateral CN.

This study demonstrates that cells in all three subdivisions of the CN (the DCN, the PVCN and the AVCN) possess cells that project directly to the CNIC. These observations are consistent with earlier findings in a range of species. In the cat, injections of horseradish peroxidase into the CNIC heavily labelled cells in all subdivisions of the contralateral CN compared to the ipsilateral CN (Adams, 1979, Oliver, 1987). In the rat, retrograde and anterograde tracing studies have shown that afferent projections to the CNIC arise from cells located in the nuclei of the ascending auditory pathway, including the PVCN and the AVCN (Beyerl, 1978, Coleman and Clerici, 1987, Fredrich et al., 2009, Kelly et al., 2009, Hatano et al., 2012). In the guinea pig, injections of different fluorescent tracers (FG, Fast Blue, FluoroRuby or Fluorescein dextran) into the CNIC resulted in retrograde labelling of cells in the DCN and VCN (Schofield and Coomes, 2005). In the mouse, labelled cells have been found in the CN following retrograde transport injections into the CNIC (Ryugo et al., 1981). Based on the above studies, we can conclude that there are axonal projections from the DCN and VCN to the CNIC.

The present study also showed that there was a topographic relationship between the FG injection site into the CNIC and the location of retrogradely labelled cells in the contralateral CN in guinea pigs and rats. Injection of the retrograde tracer FG into the dorsal region of the CNIC resulted in labelling of cells in the ventral regions of each of the three major subdivisions of the CN, particularly on the side contralateral to the FG injection. In contrast, there were more labelled cells in the dorsal regions of the contralateral DCN, PVCN and AVCN following injections into the ventral regions of the CNIC. Beyerl (1978) and Coleman and Clerici (1987), by injecting the neuronal tracer horseradish peroxidase into the dorsal and ventral areas of the CNIC of the rat, found that the dorsal area of the CNIC receives inputs from the ventral areas of the dorsal and ventral CN, whereas the ventral area of the CNIC receives inputs from the dorsal areas of the DCN and VCN. The topographic projections from the CN to the dorsal and ventral regions of the CNIC observed in the present study are similar to previous reports in several species, including cats (Adams, 1979, Oliver, 1987), guinea pigs (Schofield and Cant, 1996b) and mice (Ryugo et al., 1981). These findings are consistent with the known tonotopic organization that has been described for the CN and IC.

A variety of strategies were used in the present study to label as many VCN cells as possible that project to the IC. As it is known that injection of a retrograde tracer at a single site in the IC is not likely to spread throughout the CNIC and label all cells that project from the CN subdivisions (Beyerl, 1978, Coleman and Clerici, 1987, Schofield and Cant, 1996b, Fredrich et al., 2009), two FG injections were made in each animal to increase the number of labelled cells in the PVCN and AVCN (as illustrated in Chapter 2 Section 2.4.). Despite this, it is still not possible to determine if the injections of FG into the CNIC were able to label all the multipolar (T-stellate) cells in the PVCN and AVCN.


In the present study, we found that injection of a retrograde tracer into the CNIC labelled multipolar cells in the PVCN and AVCN. These multipolar cells, based on morphological criteria and the direction of axonal projections, have been called T-stellate cells (Osen, 1972, Adams, 1979, Oliver, 1987, Oertel et al., 1990, Ferragamo et al., 1998). Multipolar cells are primarily observed between the octopus cell area and the nerve root cells, and few scattered cells are also observed anterior to the nerve root (Osen, 1969, Brawer et al., 1974, Oertel et al., 1990, Doucet and Ryugo, 1997). These multipolar cells innervate several auditory brainstem nuclei, including the contralateral IC, the dorsal, intermediate and ventral nuclei of the LL, and some nuclei of the SOC (Osen, 1972, Adams, 1979, Ryugo et al., 1981, Cant, 1982, Oliver, 1987, Kelly et al., 1998, Schaeffer et al., 2003, Ito et al., 2008, Fredrich et al., 2009, Kelly et al., 2009, Hatano et al., 2012). This pattern of innervation is
consistent with our findings in both the guinea pigs and rats. A previous study in cats showed that multipolar cells that leave the CN via the trapezoid body show chopper response properties when categorized according their responses to sound (Smith and Rhode, 1989). These responses can be further subdivided into sustained or transient chopper responses. The morphological properties and trajectory of axons of T-stellate cells in mice are similar to the morphological properties and trajectory of axons of sustained or transient chopper cells in cats (Rhode et al., 1983, Smith and Rhode, 1989, Oertel et al., 1990). This suggests that the retrograde tracer injections into the CNIC are able to label multipolar (T-stellate or chopper) cells in the PVCN and AVCN (Osen, 1972, Roth et al., 1978, Adams, 1979, Ryugo et al., 1981, Cant, 1982, Oliver, 1987, Smith and Rhode, 1989, Oertel et al., 1990, Kelly et al., 2009, Hatano et al., 2012).

The present experiments in both guinea pigs and rats suggest that some collateral branches of MOC axons may innervate multipolar cells in the VCN, as previously shown in ultrastructural studies (Benson and Brown, 1990, Benson et al., 1996). However, the number of putative contacts was very low. The double-labelling experiments in four guinea pigs identified 21 (0.21%) multipolar (T-stellate) cells showing putative synaptic contacts, out of 9854 FG labelled cells analysed in the VCN contralateral to the FG injection site. In the five labelled rats, the MOC axonal varicosities made putative synaptic contact with only 15 (0.07%) multipolar (T-stellate) cells, out of 20551 FG labelled cells examined in the VCN contralateral to the FG injection site. Although possible synaptic contacts were found, there are a number of unresolved issues relating to these contacts. It is not known if the synaptic contacts were real or methodological artefact, what the best method for determining the presence or absence of synaptic contacts is, and whether the double-labelling experiments labelled all the MOC axonal varicosities and multipolar (T-stellate) cells in the PVCN and AVCN. These issues will not be discussed in the present chapter, but they will be addressed in the thesis discussion (Chapter 7).

The data obtained in the present study may have implications in auditory processing as the results suggest that collateral branches of MOC make possible contacts with VCN multipolar (T-stellate) cells. These cells are believed to provide excitatory input directly to the midbrain (Oliver, 1987, Smith and Rhode, 1989, Palmer et al., 1996) and are believed to be implicated in encoding complex auditory stimuli, necessary for the discrimination of speech sounds
(Rhode and Smith, 1986, Blackburn and Sachs, 1989, May et al., 1998). The present findings are in agreement with a previous study from Fujino and Oertel (2001), which recorded VCN unit responses to cholinergic agonists in brain slices of the mouse CN. They found that T-stellate cells respond to cholinergic agonists. On the basis of these findings, they suggested that T-stellate cells in the VCN are under the direct control of MOC efferents (Fujino and Oertel, 2001).

In this study, many MOC axonal varicosities were shown to make possible synaptic contact with unlabelled structures in the PVCN and AVCN. These findings could be because our injections into the IC did not result in labelling all multipolar (T-stellate) cells that project to the CNIC. It might also be because unlabelled structures in the VCN may correspond to the other group of multipolar cells that project to the contralateral CN (Adams and Warr, 1976, Cant and Gaston, 1982, Benson and Potashner, 1990, Schofield and Cant, 1996a, Doucet and Ryugo, 1997). These multipolar cells probably correspond to D-stellate cells and onset chopper cells (Smith and Rhode, 1989, Oertel et al., 1990, Palmer et al., 2003, Arnott et al., 2004). Unlabelled structures, which were observed to receive innervation from the MOC axonal varicosities, may correspond to unknown type of multipolar cells. Physiological studies using in vivo methods have also suggested that MOC axonal branches make excitatory connections with onset chopper cells (Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). The axons of onset chopper cells are shown to leave the CN via the intermediate acoustic stria and project to the contralateral CN (Smith and Rhode, 1989, Palmer et al., 2003, Arnott et al., 2004). Therefore, in Chapter (5), by injecting the neuronal tracer FG into the CN and biocytin at the centre of the floor of the fourth ventricle in both the guinea pigs and rats, we investigate whether multipolar (D-stellate or onset chopper) cells projecting to the contralateral CN receive synaptic innervation from MOC axonal varicosities.

In summary, the present study found that injections of the retrograde tracer FG into the CNIC of guinea pigs and rats label multipolar (T-stellate) cells in the PVCN and AVCN contralateral to the injection site. By combining anterograde and retrograde experiments we were able to find some limited evidence that multipolar (T-stellate) cells receive synaptic innervation from the MOC axonal varicosity in the VCN. The majority of these synapses were found on the multipolar cell bodies. These synapses were predominately located in the core
of the VCN in the guinea pigs and in the medial edge of the VCN in the rats. However, the vast majority of VCN cells labelled after FG injection into the IC, were not contacted by MOC axonal terminals. Furthermore, many MOC collateral terminals in the CN appeared to be on structures that were unlabelled and likely did not project to the IC.
Chapter 5

Anatomical Relationship between Multipolar (D-stellate) Cells and MOC Collateral Branches in the Cochlear Nucleus of Guinea Pig and Rat

5.1. Introduction

The previous anterograde and retrograde tracing experiments showed some evidence of synapses present between multipolar (T-stellate) cells projecting to the CNIC and MOC axonal varicosities in the PVCN and AVCN of guinea pigs and rats (for more details see Chapter 4). Some physiological studies have suggested that onset chopper cells that correspond to multipolar (D-stellate) cells are targeted by the MOC collateral branches (see Chapter 1, Section 1.4.3.). Multipolar (D-stellate) cells project via the intermediate acoustic stria to the contralateral VCN. Therefore, the aim of the present study was to label multipolar (D-stellate) cells in the VCN of guinea pigs and rats by injection of the retrograde tracer FG into the contralateral VCN and then investigate whether these cells receive synaptic innervation from MOC axonal varicosities. For further details of the methods see Chapters 2 and 3.

5.2. Results

5.2.1. Locations and Characteristics of FG Injection Sites

The retrograde tracer FG was successfully injected into the VCN in 11 guinea pigs and 16 rats. Three injections at different depths within the VCN were made in order to maximise the number of FG labelled cells in the auditory brainstem structures, particularly cells that project their axons to the contralateral VCN. The three FG injections into the VCN were separated in depth by 0.5mm in the guinea pigs and 0.3mm in the rats. The centre of the three injections in all animals was restricted to the VCN itself. In almost all animals, the three injection sites into the VCN were very difficult to distinguish from each other, because they typically fused together, encompassing most of the caudal and rostral regions of the VCN. The appearance of the injection sites into the VCN in both the guinea pigs and rats was similar to the injections into the CNIC described in Chapter 4. Injections into the VCN
resulted in a small central necrotic area, surrounded by a region of heavy labelled cells and a peripheral halo of lightly labelled neuropil (Figure 5.1A and 5.2A).

Figure 5-1: Photomicrographs of retrograde labelling in the auditory brainstem nuclei after a unilateral injection of FG into the ventral cochlear nucleus (VCN) in guinea pigs. (A) The FG injection site in the VCN. (B C and D) FG labelled cells in the lateral nucleus of the trapezoid body (LNTB), the ventral nucleus of the trapezoid body (VNTB) and the medial nucleus of the trapezoid body (MNTB). (E and F) FG labelled cells (arrows) in the dorsal (DCN) and ventral (VCN) cochlear nuclei. (G and H) High magnification images of FG labelled multipolar cells with their extensive dendrites in the VCN contralateral to the FG injection site. Scale bars in A-D = 200µm, in E-F = 125µm and G-H = 50µm.
While the core of the injection was within a single subdivision of the VCN, the tracer typically spread to the other subdivisions, the PVCN or AVCN. In five guinea pigs and three rats, the injection site was primarily centred in the posterior subdivision of the VCN and also spread to include the caudal region of the AVCN. In the remaining six guinea pigs and 13 rats, the injection site was mainly centred in the anterior subdivision of the VCN with some overlap into the rostral region of the PVCN. Light microscopy showed that injections into the
VCN in both the guinea pigs and rats were commonly located in central region of the VCN, but sometimes located in the medial and lateral regions of the PVCN and AVCN. In all successfully labelled guinea pigs and rats, retrograde tracer injected into the VCN did not spread into neighbouring auditory structures such as the contralateral CN, the SOC, the LL, or the IC.

In the 11 successfully labelled guinea pigs, the size of the individual FG injections varied from 263-485µm (mean = 320.9, SD = 61.4µm) in the dorso-ventral direction and from 123-255µm (mean = 212.7.9, SD = 47.6µm) in the medio-lateral direction. The extents of the FG injections in the rostro-caudal direction varied from 780-1440µm (mean = 1183.6, SD = 182.2µm). In the 16 successfully labelled rats, the size of the FG injections varied from 116-679µm (mean = 395.4, SD = 193.4µm) in the dorso-ventral direction, from 79-406µm (mean = 230.9, SD = 109.2µm) in the medio-lateral direction and from 360-1260µm (mean = 813.7.6, SD = 248.8µm) in the rostro-caudal direction.

5.2.2. Distribution of Labelled Cells in the Nuclei of the Auditory System

The FG injections into the VCN resulted in labelling of cells in several nuclei of the auditory system. The spatial distribution pattern of FG labelled cells in the guinea pigs resembled that in the rats. Injections centred into the VCN resulted in retrograde labelling of cells in the LNTB, VNTB and MNTB (Figures 5.1B-D and 5.2B-C). In all cases, labelled cells in the SOC nuclei were most abundant on the side ipsilateral to the FG injection. On both sides of the brainstem, the LNTB, VNTB and MNTB contained large multipolar cells. The location of labelled cells in the SOC region (the LNTB, the VNTB and the MNTB) is similar to that previously described in guinea pigs (Winter et al., 1989, Benson and Potashner, 1990, Shore et al., 1991), cats (Elverland, 1977, Adams, 1983a) and tree shrews (Covey et al., 1984).

Retrogradely labelled cells were also seen in the CN contralateral to the FG injection site. Injections into the VCN resulted in sparse labelling of cells in the contralateral DCN in guinea pigs and rats, which is consistent with results obtained from earlier studies in several species (Cant and Gaston, 1982, Wenthold, 1987, Schofield and Cant, 1996a). The retrogradely labelled cells in the DCN were typically found in the deep cell layer, but rarely found in the outer cell layer of the DCN. In general, FG labelled cells in the DCN gave rise to
three or more dendrites that extended from the cell bodies to the external and internal surfaces of the DCN (Figures 5.1E and 5.2D). Based on their morphological features, retrogradely labelled cells in DCN in this study could be identified as giant cells, as previously identified in other studies (Harrison and Irving, 1965, 1966b, Brawer et al., 1974, Cant and Gaston, 1982, Webster and Trune, 1982, Wenthold, 1987, Hackney et al., 1990, Schofield and Cant, 1996a).

The present study also found that labelled cells were widely scattered throughout the rostro-caudal region of the PVCN and AVCN opposite to the FG injection site (Figures 5.1F and 5.2E). This pattern of labelling was found in many of the cases where the injections spread to both subdivisions of the contralateral VCN. These findings are similar to those previously described in several species, including guinea pigs (Wenthold, 1987, Benson and Potashner, 1990, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996a), rats (Alibardi, 1998a, Doucet et al., 2009) and cats (Adams and Warr, 1976, Cant and Gaston, 1982). The distribution, shape, dendritic morphology and size of FG labelled cells in the contralateral VCN were examined, since this region was the focus of the present study.

The total number of retrogradely labelled cells projecting to the contralateral CN ranged from 60-143 in guinea pigs and from 42-201 in rats (Figures 5.3 and 5.4). In almost all animals, FG labelled cells were most abundant in the rostral PVCN and caudal AVCN, but were also found in the caudal PVCN and rostral AVCN. The location of FG labelled cells within the contralateral PVCN and AVCN did not have any obvious relationship with the VCN injection site in both the guinea pigs and rats. However, the number of FG labelled cells in the contralateral VCN was related to the site where the FG injections were centred. In some animals, labelled cells in the PVCN and AVCN contralateral to the FG injection site were fewer in comparison to others and in these animals the FG injections were not located in into the central region of the VCN, but placed into the medial or lateral regions of the VCN, resulting in a few labelled cells in the VCN opposite to the FG injection site. Figures 5.5 and 5.6 show the distribution pattern of FG labelled cells in the PVCN and AVCN after successful injection into the central region of the contralateral VCN in guinea pigs and rats.
Figure 5-3: Numbers of FG labelled cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the guinea pigs.

Figure 5-4: Numbers of FG labelled cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the rats.
The retrogradely labelled cells in the caudal and rostral regions of the PVCN and AVCN were categorized as multipolar cells, based on their morphological characteristics. Earlier studies have shown that injections into the VCN result in retrograde labelling of multipolar cells in the PVCN and AVCN contralateral to the injection site (Cant and Gaston, 1982, Wenthold, 1987, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996a, Alibardi, 1998a, Doucet et al., 2009). No other cell type was identifiable among the FG labelled cells in the PVCN and AVCN. Figures 5.1G-H and 5.2F-G show examples of large multipolar cells in the PVCN and AVCN.

There were apparent differences in the intensity of labelling of neuronal cell bodies and dendrites in both the guinea pigs and rats, although in both species the fluorescent retrograde neuronal tracer FG was employed to label the contralateral VCN cells. In guinea pigs, injections of the FG resulted in faint labelling of cell bodies in the contralateral PVCN and AVCN. In addition, the tracer FG only transported for a short distance to the primary dendrites of FG labelled multipolar cells, as shown in Figure 5.1. In rats, the cell bodies of the PVCN and AVCN were strongly labelled retrogradely with FG. This tracer transported for a long distance to the primary, secondary, or sometimes tertiary, dendrites of retrogradely labelled multipolar cells, as shown in Figure 5.1. The differences in the intensity of cells labelling in the VCN in the guinea pigs and rats might be due to interspecies variations or the tracer may not have been taken up by all cells in the VCN. It might also be because the size of the brain in guinea pigs is greater than rats, leading to transport the tracer over much longer distances.

Retrogradely labelled cells of different shapes were observed in the PVCN and AVCN contralateral to the FG injection site. At least three types of FG labelled multipolar cells could be distinguished in the VCN: rounded, polygonal and elongated cells. The occurrence of labelled rounded cell bodies increased gradually towards the caudal end of the VCN, whereas polygonal cell bodies increased progressively towards the rostral end of the VCN. Multipolar polygonal cell bodies generally had five or more dendrites, whereas round cell bodies commonly had four, and sometimes five, dendrites. Multipolar elongated cell bodies usually had three, and occasionally four, dendrites and were generally located on the border of the VCN. The most common type of FG labelled multipolar cells projecting to the opposite PVCN and AVCN was the polygonal cell body. The dendrites of FG labelled cells were long,
tapered, infrequently branched and found across much of the PVCN and AVCN. Labelled cells, which were located in the lateral region of the VCN, had dendrites that sometimes extended into the superficial layer of the granule cell region along the lateral edge of the VCN. In addition, retrogradely labelled cells, which were situated in the dorsomedial region of the PVCN, had dendrites that rarely extended into the granule cell layer.

Figure 5-5: (A-I) A series of drawings of transverse (60µm) sections of the contralateral dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 420µm) showing the location of labelled cells following the retrograde tracer FG injections into the VCN in guinea pig (GP-2015-53). Section A is most caudal; G is most rostral. Scale bar = 500µm.
Figure 5-6: (A-F) A series of drawings of transverse (60µm) sections of the contralateral dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 420µm) showing the location of labelled cells following the retrograde tracer FG injections into the VCN in rat (R-2012-28). Section A is most caudal; G is most rostral. Scale bar = 500µm.

The cell body areas of the FG labelled multipolar cells with polygonal, rounded and elongated cell bodies were measured. The cell body areas of FG labelled cells in the PVCN and AVCN in guinea pigs and rats are illustrated in Figures 5.7 and 5.8. In guinea pigs, the cell body areas of the multipolar cells ranged from 138-2071µm² (mean = 629.4, SD = 394µm², n = 600). In rats, the cell body areas of the multipolar cells ranged from 161-2430µm² (mean = 772.01, SD = 489µm², n = 600). These findings suggest that the commissural pathways that connect the right and left CN might consist of several populations of cell bodies: small and large multipolar cell bodies. This has been previously
described in several species, including the guinea pig (Wenthold, 1987), rat (Doucet and Ryugo, 2006, Doucet et al., 2009) and cat (Cant and Gaston, 1982).

Figure 5-7: The cell body areas of the retrogradely labelled multipolar cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the guinea pigs. The mean areas of FG labelled multipolar cells is 629.4µm² (SD = 394µm², n = 600).

Figure 5-8: The cell body areas of the retrogradely labelled multipolar cells in the posterior and anterior ventral cochlear nuclei following successful injections into the contralateral ventral cochlear nucleus in the rats. The mean areas of FG labelled multipolar cells is 772.01µm² (SD = 489µm², n = 600).
5.2.3. MOC Axonal Varicosities and Multipolar Cells in the Ventral Cochlear Nucleus

Double-labelling experiments were successful in labelling both the VCN and the floor of the fourth ventricle in 3 guinea pigs and 7 rats. For more details of biocytin injections at the floor of the fourth ventricle see Chapter 3. The number of retrogradely labelled multipolar cells and anterogradely labelled MOC axonal varicosities in the VCN contralateral to the FG injection site in both the guinea pigs and rats is illustrated in Tables 5.1 and 5.2. The present study examined whether putative synaptic contact between the multipolar cells and MOC axonal varicosities in the VCN contralateral to the FG injection site were present. Further details of identifying false or probably true close contacts in the VCN contralateral to the FG injection site are described in Chapter 2 (Section 2.7.).

Table 5-1
Numbers of FG and biocytin-labelled cells and medial olivocochlear (MOC) axonal varicosity in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the three successfully labelled guinea pigs

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>Total of labelled multipolar cells in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC axonal varicosity in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-2017-75</td>
<td>143</td>
<td>1750</td>
<td>237</td>
</tr>
<tr>
<td>GP-2017-76</td>
<td>86</td>
<td>2746</td>
<td>424</td>
</tr>
<tr>
<td>GP-2017-78</td>
<td>69</td>
<td>2413</td>
<td>266</td>
</tr>
</tbody>
</table>

Table 5-2
Numbers of FG and biocytin-labelled cells and medial olivocochlear (MOC) axonal varicosity in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the seven successfully labelled rats

<table>
<thead>
<tr>
<th>Experiment in rats</th>
<th>Total of labelled multipolar cells in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC axonal varicosity in the VCN contralateral to the FG injections site</th>
<th>Total of labelled MOC neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2014-28</td>
<td>201</td>
<td>198</td>
<td>173</td>
</tr>
<tr>
<td>R-2014-53</td>
<td>195</td>
<td>1265</td>
<td>338</td>
</tr>
<tr>
<td>R-2014-37</td>
<td>194</td>
<td>1504</td>
<td>241</td>
</tr>
<tr>
<td>R-2014-40</td>
<td>167</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>R-2014-32</td>
<td>164</td>
<td>702</td>
<td>184</td>
</tr>
<tr>
<td>R-2014-29</td>
<td>88</td>
<td>1672</td>
<td>402</td>
</tr>
<tr>
<td>R-2014-30</td>
<td>42</td>
<td>566</td>
<td>105</td>
</tr>
</tbody>
</table>
The presence of putative synaptic contact between the FG labelled multipolar cells and biocytin-labelled MOC axonal varicosities were considerably infrequent. Tables 5.3 and 5.4 summarise the numbers and locations of synaptic contacts between the FG labelled multipolar cells and biocytin-labelled MOC axonal varicosities in the PVCN and AVCN contralateral to the FG injection site in guinea pigs and rats. In the three double-labelled guinea pigs, no putative synaptic contacts were seen in the VCN contralateral to the FG injection site. In the seven labelled rats, putative synaptic contacts were seen only in two rats, and therefore only these animals are described below. Two multipolar cells (1.03%) were observed to make putative synaptic contacts in the rat (R-2014-37). Three multipolar cells (3.4%) were observed to make putative synaptic contacts in the rat (R-2014-29).

### Table 5-3
**Numbers and locations of putative synaptic contacts in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the three successfully labelled guinea pigs**

<table>
<thead>
<tr>
<th>Experiment in guinea pigs</th>
<th>Putative synaptic contacts on cell body</th>
<th>Putative synaptic contacts on dendrite</th>
<th>Medial edge of the VCN</th>
<th>Core of the VCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP-2017-75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GP-2017-76</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GP-2017-78</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 5-4
**Numbers and locations of putative synaptic contacts in the ventral cochlear nucleus (VCN) contralateral to the FG injection site in the seven successfully labelled rats**

<table>
<thead>
<tr>
<th>Experiment in rats</th>
<th>Putative synaptic contacts on cell body</th>
<th>Putative synaptic contacts on dendrite</th>
<th>Medial edge of the VCN</th>
<th>Core of the VCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2014-37</td>
<td>1</td>
<td>9</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-29</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>R-2014-28</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-53</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-32</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-2014-30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The anterogradely labelled MOC axonal varicosities were observed to make contact with the cell bodies and proximal, and sometimes distal, dendrites of the FG labelled multipolar cells. Figure 5.9 show examples of putative synaptic contacts between retrogradely labelled multipolar cell bodies and/or dendrites and anterogradely labelled MOC axonal varicosities in the VCN contralateral to the FG injection site. MOC axonal varicosities were seen to make
putative synaptic contacts with four cell bodies and up to nine dendrites of retrogradely labelled cells. In the rat (R-2014-37), multiple synapses were seen on the proximal or distal dendrites of two FG labelled multipolar cells. This is in contrast to the rat (R-2014-29) in which MOC axonal varicosities, when they were observed, appeared to make contact only once with a labelled multipolar cells. The majority of putative synapses (13/14) in the rat were found on cells located near the medial edge of the VCN, whereas only one contact was found on cells located in the core of the VCN. These results might be because most MOC axonal varicosities in the two rats were located in the medial edge of the PVCN and AVCN.

Figure 5-9: Light microscope showing synaptic contacts between biocytin-labelled medial olivocochlear (MOC) axonal varicosities and FG labelled multipolar (D-stellate) cells in the ventral cochlear nucleus in rats. (A) MOC axonal varicosities make putative synaptic contacts with the distal dendrite of the labelled cell. (B) High magnification image of the synapses that were shown in (A) between MOC axonal varicosities and the dendrite of the labelled cell. (C and D) MOC axonal varicosities make putative synaptic contacts with the cell bodies of the retrogradely labelled cells. Scale bars in A, C and D = 50µm, and in B = 25µm.
In all successfully labelled guinea pigs and rats, many MOC axonal varicosities were seen to make contact with VCN structures that were not labelled by the FG injection (Figure 5.10). These findings are similar to those obtained in the previous double-labelling experiments in which multipolar (T-stellate) cells projecting to the CNIC and MOC axonal varicosities were successfully labelled in the VCN of guinea pigs and rats (see Chapter 4). Since the VCN contains many distinct classes of cells that can be distinguished on the basis of anatomical, physiological and pharmacological properties, more details about the unlabelled cells in the VCN contralateral to the FG injection site are discussed below.

![Figure 5-10: Photomicrographs showing examples of putative synaptic contacts between medial olivocochlear axonal varicosities (arrows) and unlabelled ventral cochlear nucleus structures (stars) in (A) guinea pigs and (B) rats. Scale bars in A-B = 40µm.](image)

5.3. Discussion

The present study shows that injections of FG into the VCN in both the guinea pigs and rats labels cells in several locations in the auditory system. In both species, the distribution of labelled cells within the nuclei of the auditory system was similar. Labelled cells were observed bilaterally in the primary and periolivary regions of the SOC. The heaviest labelling of FG labelled cells was found in the LNTB, VNTB and MNTB ipsilateral to the injection site in the VCN, with a smaller contribution from the same nuclei on the opposite side of brainstem. This pattern of innervation is consistent with earlier results in a range of species, including guinea pigs (Winter et al., 1989, Benson and Potashner, 1990, Shore et al., 1991), cats (Elverland, 1977, Adams, 1983a) and tree shrews (Covey et al., 1984).

Following successful injection into the VCN, labelled cells were observed in the contralateral DCN, PVCN and AVCN, in agreement with the findings of studies in other species. In the
guinea pig, previous studies of retrograde tracer injection into the VCN found that contralateral retrogradely labelled cells are present in all subdivisions of the CN (Wenthold, 1987, Winter et al., 1989, Benson and Potashner, 1990, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996a). In the rat, labelled cells have also been observed in the DCN and VCN following injection of the retrograde tracer into the contralateral VCN (Alibardi, 1998a, Doucet et al., 2009). In the cat, iontophoretic injections of horseradish peroxidase into different locations in the contralateral VCN resulted in retrograde labelling of cells in the DCN and VCN (Cant and Gaston, 1982). These studies show that there are direct connections between the right and left CN.

In the present study, a few labelled cells were found in the deep layer of the contralateral DCN. Morphologically, these DCN labelled cells appear to correspond to giant cells (Harrison and Irving, 1965, 1966b, Kane et al., 1981, Webster and Trune, 1982, Hackney et al., 1990). In the guinea pig and cat, these giant cells have been shown to project to the contralateral CN (Cant and Gaston, 1982, Wenthold, 1987, Schofield and Cant, 1996a). In the present study, multipolar cells were seen scattered throughout the contralateral PVCN and AVCN in both the guinea pigs and rats. These results are in broad agreement with earlier studies showing commissural projections from one CN to the other arising from large multipolar cells in the contralateral VCN (Adams and Warr, 1976, Cant and Gaston, 1982, Wenthold, 1987, Benson and Potashner, 1990, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996a, Alibardi, 1998a, Doucet et al., 2009). The retrogradely labelled multipolar cells in this study were categorised, based on cell body shape, into three types: polygonal, rounded and elongated. The three different types of multipolar cell bodies could correspond to the categories described by Shore et al. (1992), although in the present study some differences in location were found. Shore and co-workers found that round to oval cell bodies were most abundant in the VCN contralateral to the injected CN, in contrast to the findings in the present study that showed that polygonal cells were most abundant in the VCN. A possible explanation for this difference might be that some cell bodies categorised as elongated in the present study might appear round to oval in Shore’s study due to the angle of the section (Shore et al., 1992).

The present study showed that the average area of FG labelled multipolar cells was 629.4µm² (SD = 394µm², n = 600) in the guinea pigs and 772.01µm² (SD = 489µm², n = 600)
in the rats. Doucet and Ryugo (2006), by injecting different fluorescent tracers (FG and Fast Blue) into the VCN in the rat, were also able to retrogradely labelled multipolar cells projecting to the contralateral VCN and measured the areas of their cell bodies. They found that the mean area of these labelled cells was 418µm² (SD = 140µm², n = 50). Doucet et al. (2009) found similar results in the rat using the retrograde tracer FG (mean = 419, SD = 162µm², n = 116). In the cat, Cant and Gaston (1982) also measured the areas of multipolar cells projecting to the contralateral VCN and found that the mean areas of the labelled cells was 510µm² (SD = 21µm², n = 96). Based on the above studies, injections of the retrograde tracer FG into the VCN in guinea pigs and rats in the present study were able to label large multipolar cells that project their axons to the contralateral PVCN and AVCN.

Multipolar cells projecting to the contralateral CN have been identified as D-stellate cells, on the basis of their morphological features and the direction of their axonal projections (Oertel et al., 1990, Ferragamo et al., 1998). These multipolar (D-stellate) cells project out of the CN via the intermediate acoustic stria towards the DCN and VCN on the opposite side of the brainstem (Wenthold et al., 1987, Oertel et al., 1990, Benson et al., 1996). This pattern of innervation is in agreement with the results described in the present study in both the guinea pigs and rats.

The injection strategy in the present study was designed to increase the number of labelled cells in the contralateral PVCN and AVCN by injecting the retrograde tracer FG into the VCN at different depths, see Chapter 2, Section 2.4.4. An earlier study in guinea pigs found that injection of wheatgerm agglutinin conjugated to horseradish peroxidase into the contralateral CN labelled 216-287 CN cells (Shore et al., 1992). Another study in rats found that the total number of labelled CN cells following injection of the retrograde tracer FG or fast blue into the contralateral CN ranged from 244-506 (Doucet and Ryugo, 2006). In the present study, the total number of labelled CN cells ranged from 60-143 in guinea pigs and from 42-201 in rats. In comparison to the estimated numbers obtained by others suggests that the injections in this study were able to label about 50% of the contralateral CN cells in the guinea pig (GP-2017-75) and about 40% of these cells in the rat (R-2014-28).

Interestingly, the total number of labelled cells in the contralateral VCN in the guinea pigs and rats was different following injections into the IC or VCN. Retrogradely labelled T-
stellate cells in the PVCN and AVCN were higher in number than the labelled D-stellate cells (Tables 4.1, 4.2, 5.1 and 5.2). Injections of the tracer into the IC labelled approximately 3700 cells in the contralateral VCN in the guinea pig and about 5300 cells in the rat, whilst injections into the contralateral VCN labelled only 143 VCN cells in the guinea pig and 201 cells in the rat. These results suggest that projections from the VCN to the IC are significantly larger than those projections to the contralateral VCN in both the guinea pigs and rats. Moreover, the current study shows that labelled T and D-stellate cells in the VCN in guinea pigs were lower in number than those found in rats. These results might be because the anatomical structure of the IC and CN in guinea pigs is much larger than those in rats and the retrograde tracer injections may not have filled an equivalent area of the structures, resulting in labelling a few cells projecting their axons to the contralateral VCN. However, this may be a true difference as our data are in agreement with previous studies in guinea pigs (Shore et al., 1992, Schofield and Cant, 1996b) and rats (Beyerl, 1978, Doucet and Ryugo, 2006).

In the present study, the retrograde and anterograde tracing experiments provide some evidence of the existence of small number of synapses between the biocytin-labelled MOC axonal varicosities and the FG labelled multipolar (D-stellate) cells in the VCN. By extracellular injection of horseradish peroxidase into the spiral ganglion bundle of mice, Benson and Brown (1990) and Benson et al. (1996) also found that the postsynaptic targets of MOC collateral branches in the PVCN and AVCN are varicose dendrites which are thought to originate from small cells and large dendrites which, in turn, are thought to originate from multipolar cells. In the present study, in the three successfully double-labelled guinea pigs, none of the 298 labelled multipolar (D-stellate) cells were seen to make synaptic contacts with MOC axonal varicosities. In the seven double-labelled rats, of 1051 labelled multipolar (D-stellate) cells, only 6 (0.6%) were seen to receive possible synaptic contacts from MOC axonal varicosities. Even though putative synaptic contacts between the MOC axonal varicosities and multipolar (D-stellate) cells were rarely found, a number of issues regarding the synapses in this study need to be addressed. All these issues have been mentioned in Chapter 4 (Section 4.3.), but will be discussed in more depth in the thesis discussion (Chapter 7).
In cats, Smith and Rhode (1989) showed that multipolar cells that project out of the CN via the intermediate acoustic stria show onset chopper response characteristics when classified according their responses to tones. The morphological features and course of axons of multipolar (D-stellate) cells in mice correspond to the morphological features and course of axons of onset chopper cells in cats (Smith and Rhode, 1989, Oertel et al., 1990). In guinea pigs and rats, Mulders and colleagues studied the effects of MOC collateral branches in the VCN by electrically stimulating the OC axons at the floor of the fourth ventricle whilst recording the extracellular and intracellular responses of single cells in the VCN. They suggested that collateral branches of MOC neurons make excitatory connections with onset chopper cells in the PVCN and AVCN (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). Several studies have suggested various possible functions for multipolar (D-stellate or onset chopper) cells projecting to the contralateral CN. The functions include inhibition of other cells in the DCN and VCN, and detection of spectral cues related to sound localization and detection of complex signals in noisy environments (Nelken and Young, 1994, Ferragamo et al., 1998, Verhey et al., 2003, Arnott et al., 2004, Neuert et al., 2004).

In addition to the synapses in the VCN opposite to the injection site, MOC axonal varicosities in the present study were also seen to make potential contacts with unlabelled structures in the VCN contralateral to the FG injection site. This might be because these unlabelled structures may be another population of multipolar cells projecting to the CNIC, as previously shown in Chapter 4 (Beyerl, 1978, Adams, 1979, Ryugo et al., 1981, Coleman and Clerici, 1987, Oliver, 1987, Fredrich et al., 2009, Kelly et al., 2009, Hatano et al., 2012). It could also be because the injections of the retrograde tracer did not label all multipolar (D-stellate) cells projecting to the contralateral PVCN and AVCN. Moreover, unlabelled structures might correspond to unidentified multipolar cells. Several studies, using combined retrograde transport and immunocytochemical methods, have found that the cells in the CN that project to the contralateral CN are glycinergic (Godfrey et al., 1977, Godfrey et al., 1978, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Saint-Marie et al., 1991, Kolston et al., 1992). Because it is not known whether all multipolar (D-stellate) cells were labelled in the present study, in Chapter 6, by injecting the neuronal tracer biocytin at the floor of the fourth ventricle in transgenic mice, we assess whether
glycinergic cells that may correspond to multipolar (D-stellate) cells receive synaptic innervation from MOC axonal varicosities.

In summary, the present study shows that injections of the retrogradely transported tracer FG into the contralateral VCN of guinea pigs and rats resulted in labelling of multipolar (D-stellate) cells in the VCN. Double-labelling experiments were successfully performed in three guinea pigs and seven rats. Putative synaptic contacts between MOC axonal varicosities and multipolar (D-stellate) cells projecting to the contralateral VCN were seen only in two rats. Most of the synapses were found on the dendrites of multipolar cells. These synapses were mainly located in the medial edge of the PVCN and AVCN.
Chapter 6
Anatomical Relationship between Glycinergic Cells and MOC Collateral Branches in the Cochlear Nucleus of Transgenic Mice

6.1. Introduction

In the previous double-labelling experiments, very few putative synaptic contacts were observed between MOC axonal varicosities and multipolar (D-stellate) cells in the VCN in the guinea pigs and rats (for more details see Chapter 5). It is unclear whether there are only a small number of putative synaptic contacts or whether injection of FG into the CN did not result in labelling all D-stellate cells. Therefore, an alternative method is required in which most, if not all, multipolar (D-stellate) cells should be labelled to determine if a greater number of contacts exist.

Several studies have shown that multipolar (D-stellate) cells are glycineergic (Wenthold et al., 1987, Smith and Rhode, 1989, Benson et al., 1996, Ferragamo et al., 1998, Doucet et al., 1999). Therefore, the purpose of the present study was to investigate whether glycineergic cells (which presumably include D-stellate cells) in the VCN of transgenic mice receive synaptic innervation from MOC axonal varicosities. These transgenic mice specifically express EGFP under the control of the GlyT2 gene promoter, which is a marker for glycineergic cells (Zeilhofer et al., 2005). Details of the methods used to label MOC axonal varicosities and glycineergic cells in the PVCN and AVCN of transgenic mice have been previously described in Chapters 2 and 3.

6.2. Results

6.2.1. Distribution of Labelled Cells in the Nuclei of the Auditory System

The distribution pattern of EGFP labelled cells in the SOC and CN in the transgenic mice is shown in Figures 6.1, 6.2 and 6.3 in which labelled cells were identified by fluorescence and immunohistochemical methods. A substantial number of labelled cells were seen in the SOC region. The heaviest labelling and highest density of EGFP labelled cells were found in the MNTB (Figure 6.1B). Typically, these cells had spherical or ellipsoidal shaped cell bodies, with one or two primary dendrites extending from their cell body. The cell body areas of the
EGFP labelled cells ranged from 65-225µm$^2$ (mean = 129, SD = 28.3 µm$^2$, n = 129) and the average diameter of these cells ranged from 9-19µm (mean = 14, SD = 1.9µm). Based on their location, shape and size, these cells could be principal cells, consistent with the results obtained from previous studies in the cat MNTB (Morest, 1968, 1973). Previous immunohistochemical and pharmacological studies have also shown that the principal cells of the MNTB are glycinergic (Moore and Caspary, 1983, Wenthold et al., 1987, Aoki et al., 1988, Benson and Potashner, 1990, Bledsoe et al., 1990, Henkel and Brunso-Bechtold, 1995, Friauf et al., 1999). In addition to labelling in the MNTB, EGFP labelled cells were also seen in the VNTB, LNTB, DMPO and LSO (Figures 6.1C-E). The VNTB, LNTB and DMPO contained multipolar cells, whereas the LSO contained multipolar and bipolar cells. These results suggest that cells in the nuclei of VNTB, LNTB, DMPO and LSO probably use glycine as a neurotransmitter as described in several other studies (Wenthold et al., 1987, Benson and Potashner, 1990, Friauf et al., 1999).
Figure 6-1: (A) Photomicrograph of EGFP labelled cells in some nuclei of the superior olivary complex in transgenic mice. (B and C) High magnification images of EGFP labelled cells in (B) the ventral nucleus of the trapezoid body (VNTB) and the dorsomedial periolivary nucleus (DMPO) and (C) the lateral nucleus of the trapezoid body (LNTB) and the lateral superior olive (LSO). Scale bars in A = 300µm, in B-C 200µm.
Figure 6-2: (A, B and C) Photomicrographs of EGFP labelled cells in (A) the dorsal cochlear nucleus (DCN), (B) the posterior ventral cochlear nucleus (PVCN) and (C) the anterior ventral cochlear nucleus (AVCN) in transgenic mice. The octopus cell area (OCA) of the PVCN is almost devoid of EGFP labelling, as shown in B. (D and E) High magnification images of EGFP labelled multipolar cells in (D) the PVCN and (E) the AVCN. Scale bars in A-C = 300µm, in D-E 100µm.
Figure 6-3: (A, B and C) Photomicrographs of EGFP labelled cells in (A) the dorsal cochlear nucleus (DCN), (B) the posterior ventral cochlear nucleus (PVCN) and (C) the anterior ventral cochlear nucleus (AVCN) in transgenic mice. The octopus cell area (OCA) of the PVCN is almost devoid of EGFP labelling, as shown in B. (D and E) High magnification images of EGFP labelled multipolar cells in (D) the PVCN and (E) the AVCN. (F and G) Immunoreactive labelled puncta (arrows) around unlabelled cell bodies in (F) the PVCN and (G) the AVCN. Scale bars in A-C = 200µm, in D-G = 50µm.
Labelled cells were also observed in all three major subdivisions of the CN, including the DCN, PVCN and AVCN (Figures 6.2A-C and 6.3A-C). They were most abundant in the DCN and were generally located in the fusiform and deep cell layers of the DCN, consistent with reports of high glycine levels in these areas of the DCN (Godfrey et al., 1977, Godfrey et al., 1978, Wenthold et al., 1987, Osen et al., 1990, Saint-Marie et al., 1991, Godfrey et al., 1997). Labelled cells in the fusiform cell layer were often spherical, with several spiny dendrites emanating from the cell body at many different directions. These dendrites, in some cases, extended towards the molecular cell layer of the DCN. Labelled cells in the fusiform cell layer were usually larger than those labelled in the deep cell layer of the DCN. Based on their location, shape and size, the labelled cells within the fusiform cell layer are likely to be cartwheel cells, as suggested by previous immunohistochemical studies in the guinea pig (Wenthold et al., 1987, Saint-Marie et al., 1991) and cat (Osen et al., 1990).

Small cells were also labelled in the DCN. These cells usually had oval or elongated shaped cell bodies, with one or two dendrites extending from their cell body. Small labelled cells were usually found in the deep cell layer of the DCN, but they were also found in the superficial cell layer. Based on their characteristics, these cells are likely to be tuberculoventral cells (Wenthold et al., 1987, Saint-Marie et al., 1991). Previous studies have shown that small oval or elongated (tuberculoventral) cells situated within the deep and superficial cell layers of the DCN are glycine positive cells (Wenthold et al., 1987, Osen et al., 1990, Saint-Marie et al., 1991). In line with previous studies that report that fusiform cells are unlikely to be glycinergic (Wenthold, 1987, Wenthold et al., 1987, Osen et al., 1990, Saint-Marie et al., 1991), fusiform cells in the DCN were not labelled in our study.

Labelled cells were also seen in the PVCN and AVCN. Differences in size suggest at least two populations of labelled cell bodies were present in the VCN. The first population of labelled cells consisted of small round or spherical cell bodies with average diameters of 9µm (SD = 1.3µm, n = 173) and cell body areas of 30-100µm² (mean = 69, SD = 17µm²). The other population of labelled cells consisted of large polygonal or round cell bodies with average diameters of 17µm (SD = 3.3µm, n = 173) and cell body areas of 102-363µm² (mean =188, SD = 54.6µm²). These labelled large cells commonly gave rise to three or more dendrites that left the cell body at many different angles (Figures 6.2D-E and 6.3D-E). On the basis of their morphological properties these cells could be characterized as multipolar cells, as
previously described in many species, including rats (Harrison and Irving, 1965, 1966b), cats (Osen, 1969), guinea pigs (Hackney et al., 1990) and mice (Webster and Trune, 1982).

While EGFP labelled cells were commonly observed near the auditory nerve root, the octopus cell area of the PVCN (Osen, 1969) was almost devoid of EGFP labelling. This agrees with an earlier study by Wickesberg et al. (1991) using immuno-labelling for glycine. Labelled cells were most abundant in the caudal AVCN and less frequently found in the PVCN and the rostral AVCN. These cells were small and spherical, and were most abundant in the granule cell layer of the AVCN. The distribution and morphological properties of cell bodies in the VCN in the present study correspond largely to the distribution and morphological features of glycinergic cells described by others (Altschuler et al., 1986, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Friauf et al., 1999, Zeilhofer et al., 2005). The locations of EGFP labelled small and large cell bodies in the PVCN and AVCN are shown in Figure 6.4.

![Figure 6-4: (A-E) A series of drawings of transverse (40µm) sections of the dorsal (DCN) and ventral (VCN) cochlear nuclei (distance between sections approximately 280µm) showing the location of labelled large (x) and small (o) cells in the posterior (PVCN) and anterior (AVCN) in a transgenic mouse (M-2016-12). Section A is most caudal; E is most rostral. Scale bar = 200µm.](image)

The perimeters of unlabelled cell bodies were frequently covered with labelled puncta, which probably represent immunoreactive presynaptic terminals (Figures 6.3F-G). These
findings are similar to those previously reported in guinea pigs (Altschuler et al., 1986, Wenthold, 1987, Wenthold et al., 1987) and rats (Friauf et al., 1999). In the DCN, unlabelled cell bodies within the fusiform and deep cell layers often showed such punctate labelling around the cell body. A similar subcellular pattern of labelled puncta was also found in the PVCN and AVCN, with the heaviest labelling in the AVCN. Labelled puncta, however, were rare or absent in the octopus cell area of the PVCN and the granule cell layer of the PVCN and AVCN.

6.2.2. MOC Axonal Varicosities and Glycinergic Cells in the Ventral Cochlear Nucleus

A midline injection of the tracer biocytin at the floor of the fourth ventricle (where MOC axons are grouped in a tight bundle between the facial genua) was successfully performed in one transgenic mouse. In this animal, the injections resulted in labelling of MOC axons and their en passant and terminal varicosities in the VCN (for more details of this labelling see Chapters 2 and 3). The MOC en passant and terminal varicosities were primarily seen in the granule cell layer, and a few scattered axonal varicosities were also seen at the medial edge and core of the PVCN and AVCN. In the only labelled transgenic mouse, the total number of bilaterally labelled MOC en passant and terminal varicosities in the PVCN and AVCN was 674.

In the one successfully labelled transgenic mouse, putative synaptic contacts between the EGFP labelled cells and anterograde labelled MOC axonal varicosities in the VCN were examined bilaterally. In this animal, EGFP labelled cells were identified by immunohistochemical methods instead of fluorescence techniques. This was because EGFP fluorescent labelling completely disappeared after immuno-labelling of the biocytin using DAB amplification. Therefore, an anti-GFP antibody was used to detect the EGFP labelled cells in the VCN. Since the distribution pattern of EGFP labelled cells using immunohistochemical methods is largely compatible with the distribution pattern of fluorescently labelled cells in the VCN of mice, immunohistochemical methods were used in the present study to identify the EGFP labelled cells in the PVCN and AVCN.

Using this method there was no evidence of close contacts between the EFGP labelled cells and MOC axonal varicosities in the PVCN and AVCN. These results might be due to the
presence of small number of MOC en passant and terminal varicosities in the medial edge and core of the VCN, where many of the EGFP labelled cells were found. Figures 6.5 and 6.6 show examples of the absence of contact between the EGFP labelled cell and MOC axonal varicosity. The MOC axonal varicosities, however, were observed to make possible contact with unlabelled structures (Figures 6.5B-C and 6.6B-C). The possible identity of these unlabelled structures is discussed in more detail in Section 6.3. below.
Figure 6-5: (A, B and C) A series of photomicrographs showing the absence of putative synaptic contact between the EGFP labelled cells (thick arrows) and medial olivocochlear (MOC) axonal varicosities (thin arrows) in the ventral cochlear nucleus in a transgenic mouse. The different panels are the same section at different focal planes. (A) EGFP labelled cells are focused in a slice in which MOC axonal varicosities are not. (B and C) MOC axonal varicosities are focused in a slice in which EGFP labelled cells are not. In such example, counting was not performed, as both structures are unfocused in the same slice. The MOC axonal varicosities are shown to make putative synaptic contact with unlabelled structures (stars), as shown in B and C. Scale bars in A-C = 50µm.
Figure 6-6: (A, B and C) A series of photomicrographs showing the absence of putative synaptic contact between the EGFP labelled cell (thick arrows) and medial olivocochlear (MOC) axonal varicosities (thin arrows) in the ventral cochlear nucleus in a transgenic mouse. The different panels are the same section at different focal planes. The MOC axonal varicosities are shown to make putative synaptic contact with unlabelled structures (stars), as shown in B and C. Scale bars in A-C = 50µm.
6.3. Discussion

The present results show that the spatial distribution pattern of EGFP labelled cells in the SOC nuclei and the CN auditory structures in the transgenic mice used in this study are compatible with results obtained from previous studies (Godfrey et al., 1977, Godfrey et al., 1978, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Saint-Marie et al., 1991, Kolston et al., 1992, Zeilhofer et al., 2005). These studies showed that some nuclei of the brainstem (including the MNTB, VNTB, LNTB, DMPO and LSO, DCN, PVCN and AVCN) contain high levels of glycine positive cells.

In the present study, many of the EFGP labelled cells were found in the MNTB. Morphologically these MNTB cells may correspond to principal cells which have been described in the cat by Morest (1968, 1973). Immunohistochemical studies using antibodies to the inhibitory neurotransmitter glycine have also shown that MNTB principal cells have intense staining for glycine, which is consistent with our findings (Moore and Caspary, 1983, Wenthold et al., 1987, Aoki et al., 1988, Benson and Potashner, 1990, Bledsoe et al., 1990, Henkel and Brunso-Bechtold, 1995, Friauf et al., 1999).

Moreover, EGFP labelled cells were present in the LSO, VNTB, LNTB and DMPO. These observations are consistent with earlier studies that showed that the LSO, VNTB, LNTB and DMPO contain glycinergic cells. In the guinea pig, Wenthold et al. (1987) used antibodies selective for glycine to study the distribution pattern of glycinergic cells in the nuclei of the SOC. In addition to heavy labelling of cell bodies in the MNTB, they also found a few labelled cells scattered in the LSO nucleus, suggesting that these cells are glycinergic (Wenthold et al., 1987). Benson and Potashner (1990), by injecting the neuronal tracer [3H]glycine into the CN of the guinea pig, found bilateral retrogradely labelled cells in the periolivary regions of the SOC (including the VNTB, LNTB and DMPO), suggesting that cells in the VNTB, LNTB and DMPO that project to the CN probably use glycine as a transmitter.

The present study also found several populations of EGFP labelled cells in the DCN and VCN. In the DCN, the EGFP labelled cells were usually located in the fusiform and deep cell layers, where the highest level of glycine has been previously described (Godfrey et al., 1977, Godfrey et al., 1978). Labelled cells in the fusiform cell layer could correspond to the
cartwheel cells described in this region in the rat and mouse, and the deep cell layer cells could correspond to the small cells described in this region in the cat (Lorente de Nó, 1933, Brawer et al., 1974, Zhang and Oertel, 1993a). Several studies have shown that cartwheel cells and small cells in the DCN of guinea pigs can be consistently labelled with anti-glycine antibody, indicating that these cells are glycinergic (Wenthold et al., 1987, Osen et al., 1990, Saint-Marie et al., 1991). Saint-Marie et al. (1991), by injecting horseradish peroxidase into the AVCN of the guinea pig, labelled small cells in the deep cell layer of the DCN. Nearly all (96%) of the horseradish peroxidase labelled cells in the deep cell layer were also positive for glycine, suggesting that projections from the deep cell layer of the DCN to the AVCN are formed mainly by glycinergic cells (Saint-Marie et al., 1991).

Large and small cells were also seen in the PVCN and AVCN, while only small cells were seen in the granule cell layer. Immunocytochemical experiments have demonstrated that large and small cells can be labelled with antibodies against glycine conjugates, suggesting that these cells are probably glycinergic (Wenthold, 1987, Wenthold et al., 1987, Osen et al., 1990). An earlier study, using combined retrograde transport and immunocytochemical methods, found that in the guinea pig the cells in the VCN that project to the contralateral CN are immunoreactive for glycine, indicating that the crossed CN pathway is possibly glycinergic (Wenthold, 1987). Other studies have also reported that contralateral acoustic stimulation of the crossed CN pathway is mainly inhibitory and this inhibition is significantly antagonized by the simultaneous application of strychnine, suggesting that glycine could be the neurotransmitter of this pathway (Pirsig et al., 1968, Young and Brownell, 1976, Wenthold and Martin, 1984).

Cant and Gaston (1982) studied the terminal distribution of the crossed fibres in the CN of cats. They injected the anterograde axonal transport of horseradish peroxidase-labelled wheat germ lectin into the CN and found that the input of this pathway to the contralateral CN was relatively sparse (Cant and Gaston, 1982). These results suggest that many of the glycinergic synapses in the CN, based on localization of the glycine postsynaptic receptor and glycine immunoreactivity staining, are not mostly due to the crossed CN pathway (Cant and Gaston, 1982, Altschuler et al., 1986, Wenthold et al., 1987). In the guinea pig, combined retrograde transport and immunocytochemical labelling experiments have also shown that most of the glycinergic synapses in the PVCN and AVCN originate from the small
glycinergic cells in the DCN (Wenthold et al., 1987). This suggests that glycinergic terminals in the PVCN and AVCN may arise from the small cells in the DCN as well as from the crossed CN pathway (Cant and Gaston, 1982, Altschuler et al., 1986, Wenthold et al., 1987).

The distribution and morphological properties of large multipolar cells in this study appear to correspond to commissural cells which have been shown to project to the contralateral CN in the cat (Adams and Warr, 1976, Cant and Gaston, 1982), rat (Doucet and Ryugo, 1997) and guinea pig (Benson and Potashner, 1990, Schofield and Cant, 1996a). Commissural cells are a group of large multipolar cells that project to the contralateral CN. On the basis of their morphological features, axonal trajectories and physiological characteristics, these large multipolar cells probably correspond to D-stellate cells and onset chopper cells (Scharf et al., 1987, Smith and Rhode, 1989, Oertel et al., 1990, Shore et al., 1992, Palmer et al., 2003, Arnott et al., 2004). These multipolar cells have local axonal collaterals in the VCN and the DCN, and their axons extend through the intermediate acoustic stria and terminate in the contralateral DCN and VCN (Scharf et al., 1987, Smith and Rhode, 1989, Oertel et al., 1990, Shore et al., 1992, Palmer et al., 2003, Arnott et al., 2004). Physiological and pharmacological evidence suggests that multipolar (D-stellate or onset chopper) cells provide glycinergic inhibitory input to the contralateral and ipsilateral CN circuitry, suggesting that these cells are glycinergic and inhibitory (Wenthold et al., 1987, Smith and Rhode, 1989, Benson et al., 1996, Ferragamo et al., 1998, Doucet et al., 1999).

Previous studies have suggested that glycine is an important inhibitory neurotransmitter in the central nervous system (Werman and Aprison, 1966, Werman et al., 1967, Curtis and Johnston, 1974, Aprison et al., 1975, Young and McDonald, 1983, Harvey et al., 2000, Imboden et al., 2001, Nguyen et al., 2001, Lynch, 2004, Grudzinska et al., 2005, Young-Pearse et al., 2006). The inhibitory feedback mechanism provided by glycinergic interneurons in the spinal cord and brainstem is thought to play a crucial role in the control of motor rhythm generation during movement and in the coordination of spinal reflex activity (Grillner et al., 1998, Legendre, 2001). Glycine-mediated neurotransmission is also believed to play an important role in the processing of auditory information through the CN, the SOC and the IC (Wenthold and Hunter, 1990) and in the processing of optical information in retinal ganglion cells (Han et al., 1997, Protti et al., 1997).
Labelled puncta, presumably representing axons terminals, were commonly found around immunonegative cell bodies in the dorsal and ventral subdivisions of the CN. These puncta were most abundant in the fusiform and deep cell layers of the DCN and in the AVCN, whereas there was a paucity of labelled structures in the octopus cell area of the PVCN and the granule cell layer of the VCN. These observations are in agreement with previously published results describing glycine immunoreactivity in the CN of guinea pigs (Altschuler et al., 1986, Wenthold, 1987, Wenthold et al., 1987) and rats (Friauf et al., 1999) and mice (Wickesberg et al., 1991).

In the one transgenic mouse that was successfully double-labelled in this study, the total number of the labelled MOC axonal varicosities in the VCN was significantly lesser than in the one successfully labelled wild-type mouse that was described in Chapter 3. This suggests that injection of the neuronal tracer at the floor of fourth ventricle in the transgenic mouse only labelled a small population of the MOC system. In the one double-labelled transgenic mouse, there was no evidence of putative synaptic contact between the EGFP labelled cells and the anterogradely labelled MOC axonal varicosities in the PVCN and AVCN. These findings could be because the anterogradely labelled MOC axonal varicosities were sparse in the medial edge and core of the PVCN and AVCN, making any actual close contact between the MOC axonal varicosities and EGFP labelled cells rare. If MOC axonal varicosities preferentially target D-stellate cells, it would have been expected that at least some putative synaptic contacts on EGFP positive cells would have been observed.

Some MOC axonal varicosities were observed making possible contact with VCN structures that were not labelled with EGFP. Some of these unlabelled structures may correspond to the other population of multipolar cells (which presumably include T-stellate cells or chopper cells) that project to the contralateral and ipsilateral IC (Osen, 1972, Adams, 1979, Ryugo et al., 1981, Oliver, 1987, Smith and Rhode, 1989, Oertel et al., 1990, Palmer et al., 1996, Kelly et al., 2009, Hatano et al., 2012). Unlike D-stellate cells, multipolar (T-stellate or chopper) cells are thought to provide excitatory input to the midbrain, suggesting they are unlikely to be glycinergic (Oliver, 1987, Smith and Rhode, 1989, Palmer et al., 1996, Alibardi, 1998a, 2001). Close contact between multipolar (T-stellate) cells and MOC axonal varicosities were occasionally seen (Chapter 4), which is consistent with the study of Fujino
and Oertel (2001) involving brain slices of the mouse CN (for more details see Chapter 1, Section 1.4.3.).

In summary, the present study shows that transgenic mice which express EGFP under the control of the GlyT2 gene promoter can be successfully used in identifying glycinergic cells in the nuclei of the auditory structures. Several populations of cells were labelled in the SOC nuclei and the CN auditory structures, suggesting that these cells may use glycine as a neurotransmitter. Using double-labelling, no evidence was found for the existence of close contacts between MOC axonal varicosities and EGFP (glycinergic) labelled cells. The anterograde labelled MOC varicosities, however, were shown to make close contact with unlabelled structures, which may correspond to multipolar (T-stellate) cells or other types of cells in the PVCN and AVCN.
Chapter 7
General Discussion and Conclusions

7.1. Aims of the Thesis

This study aimed to evaluate the anatomical connectivity of the MOC collaterals in the VCN of several species. To identify which neural cell types in the VCN receive synaptic innervation from the MOC collaterals, double-labelling experiments were performed in guinea pigs and rats that combined retrograde FG labelling using injections in contralateral IC or CN (thought to selectively label T-stellate cells or D-stellate cells, respectively) with anterograde biocytin labelling of MOC collaterals. In addition, experiments were performed in transgenic mice, which express EGFP under the control of the promoter of the GlyT2 gene, to assess whether glycinergic cells in CN receive synaptic input from the MOC collaterals.

7.2. Summary of Findings

The present study shows that combined retrograde and anterograde tracing can be used to label multipolar cells and MOC collaterals in the VCN (see Chapters 3, 4 and 5). Although many MOC axonal terminals and varicosities were labelled in the PVCN and AVCN, as well as many multipolar cells, only very small numbers of putative synaptic contacts were observed. Most of these putative synapses were found on cell bodies, with some on the dendrites. The putative synapses between multipolar cells and MOC varicosities were commonly located at the medial edge of the PVCN and AVCN, but some were also found in the core of these nuclei. These results suggest that MOC axonal collaterals only provide very limited input to CN cells projecting to the contralateral CN and IC (see Chapters 4 and 5, Figures 4.10 and 5.9). In addition, although the data were limited, our results showed no close apposition between MOC collaterals and glycinergic cells in mouse CN.

7.3. Technical Considerations

The results and subsequent interpretation are subject to a number of possible technical considerations. First, our initial attempts using immunohistochemical staining of FG resulted in only weak labelling of somata and dendrites and in particular the limited visualisation of
the dendritic trees was considered insufficient to allow thorough analysis. Hence the
decision was made to rely on the fluorescent signal alone for analysis of putative synaptic
contacts. While this did result in better revelation of the dendritic arbores, it was subject to
fading which may have reduced the number of contacts observed.

Second, retrograde tracer injections will not have labelled all projecting cells within each
animal. This would possibly lead to an underestimate of the number of synaptic contacts.
Indeed, the topographic relationship of labelled cells in CN with depth of IC injection
indicates that injections into the IC in the present study did not cover the entire anatomical
structure, and therefore did not label all multipolar cells projecting from the VCN to the IC in
every animal (see Chapter 4, Section 4.2.2.). These topographic projection results are in
agreement with previous reports in several species, including cats (Adams, 1979, Oliver,
1987), guinea pigs (Schofield and Cant, 1996b) and mice (Ryugo et al., 1981). Similarly, the
differences in the total number of FG labelled multipolar cells in the VCN after injections
into the central, lateral and medial regions of the contralateral VCN also suggest that the
tracer did not label all contraterally projecting cells in each animal (see Chapter 5, Section
5.2.2., Figures 5.3 and 5.4). It is therefore possible that some of the unlabelled cells
contacted by the MOC collaterals, could still be cells projecting to contralateral IC or CN.

Indeed, results from this thesis may suggest that labelling more cells will result in the
identification of more putative synaptic contacts since a larger number of close appositions
were observed between MOC axonal varicosities and the large number of cells projecting to
IC than with the smaller number of cells that were labelled projecting to contralateral CN.
However, the percentage of cells projecting to contralateral CN that were shown to receive
putative synaptic contact by MOC collaterals was higher than that observed for cells
projecting to contralateral IC. Regardless, even in our most successful animals the numbers
and percentage of innervation were extremely low.

Third, another possible explanation for the low number of putative synaptic contacts
observed in this study may be that injections of the tracer biocytin at the floor of the fourth
ventricle label only the axons of crossed MOC neurons and their collateral branches in the
CN. This notion is supported by the lack of labelling of LOC neurons which have a similar
pattern of trajectories as the uncrossed MOC axons (Vetter and Mugnaini, 1992, Warr et al.,
Therefore, it seems likely that the results of the thesis may be limited to the collateral branches of the axons of crossed MOC neurons. However, several studies have shown that the number of crossed MOC axons is significantly more than the uncrossed MOC axons. The ratio of crossed to uncrossed MOC axons is approximately 2:1 in guinea pigs, mice and rats and 3:1 in cats (Warr, 1975, 1980, Robertson et al., 1987c, Vetter and Mugnaini, 1992, Brown and Levine, 2008). Thus, injections of the tracer biocytin could result in labelling a large proportion of the axons of MOC neurons, particularly the crossed axons, and their collateral branches in the CN (see Chapter 3). However, although the number of MOC neurons labelled in guinea pigs was lower than found in some earlier studies using intracochlear injections (Robertson et al., 1987b), in some rats the numbers were actually larger than reported by others White and Warr (1983) (Chapter 3, Section 3.3.). The problem of reliability of MOC labelling was most extreme in the mouse. In one animal numbers of neurons were similar as reported by others Campbell and Henson (1988) (Chapter 3, Section 3.3.) but in the one successfully double-labelled animal, only small numbers of labelled MOC neurons were found (see Chapter 6).

Despite these labelling limitations for both FG and biocytin, it seems unlikely that large numbers of contacts would be missed in all animals if they existed. Indeed, what is striking in the results in all animals is the large number of biocytin-labelled varicosities and terminals in apparent contact with unlabelled cells as well as the large number of FG labelled cells that are close to these varicosities and terminals but without showing close apposition. Hence, it is likely that the small number of putative contacts seen is a reflection of the true situation.

Fourth, it cannot be said that close appositions, as described in the results, are in fact true synaptic contacts. This could only be established with certainty using electron microscopy (EM). The very small numbers of putative contacts seen, mean that such a study would be extremely difficult. In addition, EM verification of true synaptic contacts would, if anything, be likely to reduce the estimate of the number of synapses on retrogradely labelled cells. To reduce the estimate of the number of synapses on retrogradely labelled cells, recent studies of three-dimensional reconstructions from serial ultrathin sections have demonstrated that real synaptic contacts between adjacent neuronal elements are much more scarce than what their sheer proximity would suggest (Kasthuri et al., 2015, Ostroff and Zeng, 2015).
7.4. Interpretation and Implications

On balance therefore, the data do not provide strong support for innervation of cells projecting to contralateral VCN or IC by MOC collaterals in guinea pigs and rats. In addition, no evidence was found in mouse for specific contacts between MOC collaterals and glycinergic cells in VCN. This raises questions about conclusions drawn by several researchers using a range of different techniques on the possible targets of the MOC collaterals.

Neuroanatomical EM studies have shown that the postsynaptic targets of MOC collateral branches in the VCN are multipolar cells, not further defined (Benson and Brown, 1990, Brown and Benson, 1992, Benson et al., 1996). Synaptic contacts with excitatory characteristics were described on somata and proximal dendrites of multipolar cells. In addition, they described synaptic contacts on small unidentified cells. Multipolar cells in CN are a highly diverse group of cells which are thought to have different projection patterns (Osen, 1972, Adams and Warr, 1976, Beyerl, 1978, Roth et al., 1978, Adams, 1979, Cant, 1982, Cant and Gaston, 1982, Coleman and Clerici, 1987, Oliver, 1987, Wenthold, 1987, Wenthold et al., 1987, Benson and Potashner, 1990, Shore et al., 1991, Shore et al., 1992, Schofield and Cant, 1996b, Fredrich et al., 2009, Kelly et al., 2009, Hatano et al., 2012).

Fujino and Oertel (2001) suggested that collateral branches of MOC axons in the VCN make excitatory cholinergic connections with a particular subgroup of multipolar cells, T-stellate cells, rather than D-stellate cells. They investigated the action of cholinergic agonists on multipolar cells in slices of the neonatal mouse VCN and found that application of cholinergic agonists resulted in excitatory effects on T-stellate cells, and no effect on D-stellate cells. However, although a lack of effect on the D-stellates is consistent with the data in this thesis, the effects observed on T-stellate are in apparent conflict with the present data. There are several technical limitations to the study of Fujino and Oertel that may mean their results should be interpreted with some caution. First, bath application of cholinergic agonists may not mimic direct MOC activation on the cells recorded and secondly, other elements of brainstem circuitry are cholinergic as well as the MOC collateral, and an effect observed from cholinergic agonists may not be necessarily representative of an effect of the MOC collaterals (Sherriff and Henderson, 1994).
Other researchers investigating the targets of the MOC collaterals used electrophysiological criteria to define cell types in VCN in vivo. Mulders and colleagues studied the effects of MOC activation on the responses of single neurons in the VCN in guinea pigs and rats (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). Using extracellular and intracellular recordings in the VCN, Mulders et al., (2002, 2003, 2007, 2009) found that onset-like cells and in particular onset choppers showed excitatory effects following electrical stimulation of MOC axons. Chemical destruction of OHCs by kanamycin or cholinergic blockade in the cochlea by strychnine, which eliminate the peripheral effects of the MOC activity, still led to excitation of some onset-like cells in the VCN (Mulders et al., 2002), suggesting a direct effect via MOC collaterals. On the other hand, chopper cells in the VCN showed either no response to electrical shocks to the MOC axons or IPSPs (Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009). These data led to their hypothesis that the MOC collaterals provide excitatory direct input to the onset-chopper cells. If it is assumed that onset choppers correspond to D-stellate cells then these data are in conflict with the data obtained by Oertel and colleagues as well as the neuroanatomical results presented in this thesis.

What is the evidence that onset choppers are D-stellate cells and project to contralateral VCN? And conversely, how strong is the evidence that chopper cells are T-stellate and project to the contralateral IC? It is well known that projections exist from multipolar cells in VCN to contralateral IC, and to ipsilateral and contralateral VCN and DCN (Adams, 1979, Smith and Rhode, 1989, Shore et al., 1992, Schofield and Cant, 1996a, 1996b). Oertel and colleagues define T-stellates as multipolar cells with an axon that exits the CN via the trapezoid body, and D-stellate cells as those multipolar cells whose axons project out of the CN via the intermediate acoustic stria (Oertel et al., 1990, Oertel and Fujino, 2001). However, in the mouse slice preparations used axons could not be traced to the contralateral IC or CN.

Smith and Rhode (1989) showed in the cat that the axon of onset choppers exited the CN via the intermediate acoustic stria whereas axons of sustained choppers exited via the trapezoid body suggesting that the former response type corresponds to D-stellate and the latter to T-stellate cells. However, data on the precise targets of these two cell types are limited. Palmer et al. (2003) confirmed the previous results that chopper cells project out via
the trapezoid body but did not reveal their final target. Their study (described in Arnott et al., 2004) also showed an extensive axon trajectory of one onset chopper which was followed as far as the contralateral DCN but which then faded out. Smith et al. (2005) also attempted to trace the axon trajectories of onset choppers. Two out of 8 filled axons were traced to contralateral CN but again faded quickly and their final destination in CN was not revealed. In addition, two labelled axons followed a different trajectory, one coursing back to the ipsilateral CN and one coursing to MSO. There are no papers that show the axon trajectory of chopper cells all the way to the contralateral IC. However, there are data available showing that type I multipolar cells in VCN form a strong projection to the contralateral IC (Alibardi, 1998b). Type I multipolar cells are thought to correspond to T-stellate cells (Cant, 1981, Smith and Rhode, 1989, Alibardi, 1998b). However, Cant and Benson (2003) suggest that not all type I multipolar cells project to contralateral IC and some may provide input to trapezoid and lemniscal nuclei.

Taken together these data do suggest that at least some of the multipolar cells in VCN that correspond to onset choppers project to contralateral VCN and that some that correspond to chopper cells project to contralateral IC. However, it remains unknown whether all onset choppers and all chopper cells project to contralateral VCN and IC, respectively. Indeed, electrophysiological data using antidromic stimulation from Needham and Paolini (2003) may suggest that some of the onset choppers do not project to contralateral CN. This is in line with the anatomical data from Smith et al. (2005) that showed that two of the 8 onset choppers in their sample had an axon that did not terminate in contralateral CN. The possibility should therefore be considered that by using retrograde tracers in VCN and in IC we may not have labelled the relevant onset choppers or chopper cells that receive MOC collateral input.

Similarly, the fact that we did not find any evidence for contacts between MOC collaterals and glycinergic cells in transgenic mouse, is in agreement with the absence of cholinergic responses in D-stellate cells reported by Fujino and Oertel (2001). Wenthold (1987) showed evidence in guinea pig of large glycinergic VCN cells that project to the contralateral CN. Hence the conventional wisdom is those multipolar cells projecting to contralateral VCN are glycinergic and D-stellate. However there is no direct evidence that onset choppers are glycinergic. The demonstration of excitatory effects of MOC stimulation in onset choppers
(Mulders et al., 2002, Mulders et al., 2003, Mulders et al., 2007, Mulders et al., 2009) may not therefore be in conflict with the results of the glycinergic experiments in this thesis.

7.5. Suggestions for Future Research

The results of the present study are somewhat inconclusive, and there are a number of improvements that could be suggested. Future studies could be directed towards double-labelling using a more efficient and permanent retrograde tracer. While the FG tracer is thought to be one of the most effective retrograde axonal transport tracers and has been used in several neuroanatomical studies to label the neuronal cell body and its dendrites (Lanciego et al., 1998a, Lanciego et al., 1998b, Köbbert et al., 2000, Mulders and Robertson, 2000b, 2004), there are a number of other retrograde tracers that could be used, such as cholera toxin subunit B. This latter tracer has been shown to be effective in terms of selective retrograde transport and neuronal labelling (Bruce and Grofova, 1992, Vetter et al., 1993, Köbbert et al., 2000). Moreover, numerous permanent immunohistochemical protocols are available for the cholera toxin subunit B tracer. Such protocols could be beneficial in future studies because the morphological features of the cells would not fade during microscopic examination (Bruce and Grofova, 1992, Vetter et al., 1993, Köbbert et al., 2000).

In the current study, a simple and fast method was used to label the axons of MOC neurons and their collateral branches in the CN (see Chapter 3). However, this method is most likely to label only the axons of crossed MOC neurons in the PVCN and AVCN. Indeed, there are other methods that can be used to label the MOC axonal varicosities in the VCN, including injection of retrograde axonal tracer into the intraganglionic spiral bundle of the cochlea (Brown et al., 1988, Benson and Brown, 1990, Brown et al., 1991, Brown and Benson, 1992, Brown, 1993, Benson et al., 1996) or injection of anterograde tracer into the nuclei of origin of MOC neurons (Warr and Beck, 1996). The latter method is non-specific, since it would also label non-MOC projections to the CN arising from the VNTB and RPO nuclei located in the SOC. Although cochlear injections of retrograde axonal tracers are methodologically very difficult and require long survival times for retrograde transport, this method can label the axons of crossed and uncrossed MOC neurons and their projections in the VCN. If the biocytin injections were supplemented with injections into the intraganglionic spiral bundle
and a double-labelling paradigm was attained, such study may assist to label the axons of both crossed and uncrossed MOC neurons and determine the specific cell target of MOC collateral branches in the CN.

Regarding the glycinergic investigations in this thesis, it is evident that more experiments are required, since in the one double-labelled animal, only a small number of MOC neurons was labelled. In addition, it is still not known whether all glycinergic cells in the VCN project to the contralateral PVCN and AVCN and whether all contraterally projecting cells are glycinergic. Therefore, injection of retrograde tracer into the contralateral VCN of transgenic mice could assist in answering this question.

Finally, in the present study, we labelled multipolar VCN cells that project to contralateral PVCN and AVCN. However, multipolar cells in the VCN have also been reported to project their axons into the ipsilateral and contralateral DCN (Adams, 1983b, Shore et al., 1992, Doucet and Ryugo, 1997). It should therefore be investigated whether these DCN projecting multipolar cells are in fact the targets of MOC collaterals.

7.6. Conclusion

The results of the current study suggest that MOC collateral branches provide very limited input to cells projecting to contralateral IC or VCN. Therefore, the data in this thesis do not resolve the apparent conflicts in the literature regarding the targets of MOC collaterals. The data also suggest that numerous axonal terminals of MOC neurons make synaptic contact with unlabelled structures in the PVCN and AVCN. The identity of these unlabelled structures remains unknown. Further research is required to determine which VCN cells are significantly innervated by the collateral branches of MOC neurons.
References


Cant NB, Casseday JH (1986) Projections from the anteroventral cochlear nucleus to the lateral and medial superior olivary nuclei. Journal of Comparative Neurology 247:457-476.


Huffman RF, Covey E (1995) Origin of ascending projections to the nuclei of the lateral lemniscus in the big brown bat, Eptesicus fuscus. Journal of Comparative Neurology 357:532-545.


Lindsey BG (1975) Fine structure and distribution of axon terminals from cochlear nucleus on neurons in the medial superior olivary nucleus of the cat. Journal of Comparative Neurology 160:81-104.


Warr WB (1975) Olivocochlear and vestibular efferent neurons of the feline brain stem: their location, morphology and number determined by retrograde axonal transport and acetylcholinesterase histochemistry. Journal of Comparative Neurology 161:159-182.


