Targets of Olivocochlear Collaterals in Cochlear Nucleus of Rat and Guinea Pig

Running title: Targets of Olivocochlear Collaterals

Ahmaed Baashar,1,2, Donald Robertson,1 Nathanael James Yates,4 and Wilhelmina Henrica Antonia Maria Mulders1,3*

1The Auditory Laboratory, School of Human Sciences, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia. 2Department of Anatomy, College of Medicine, King Saud bin Abdulaziz University for Health Sciences, Jeddah, Saudi Arabia. 3Ear Science Institute Australia, the Ralph and Patricia Sarich Neuroscience Research Institute, 8 Verdun St, Nedlands WA 6009, Western Australia, 6008, Australia. 4Preclinical Intensive Care Research Unit, School of Human Sciences, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia.

Email:

Ahmaed Baashar: ahmaed.baashar@research.uwa.edu.au
Donald Robertson: don.robertson@uwa.edu.au
Nathanael Yates: nathanael.yates@uwa.edu.au
Wilhelmina Mulders: helmy.mulders@uwa.edu.au

*Corresponding author: The Auditory Laboratory, School of Human Sciences, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia. Phone: +61 08 64883321 Fax: +61 08 64881025

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/cne.24681

© 2019 Wiley Periodicals, Inc.
Received: Nov 02, 2018; Revised: Mar 06, 2019; Accepted: Mar 07, 2019

This article is protected by copyright. All rights reserved.
Acknowledgements

The author (A Baashar) is a recipient of a postgraduate scholarship from King Saud bin Abdulaziz University for Health Sciences. This work was supported by grants from the National Health and Medical Research Council, the Medical Health and Research Infrastructure Fund (Australia) and The University of Western Australia.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.
Abstract

Descending auditory pathways can modify afferent auditory input en route to cortex. One component of these pathways is the olivocochlear system which originates in brainstem and terminates in cochlea. Medial olivocochlear (MOC) neurons also project collaterals to cochlear nucleus and make synaptic contacts with dendrites of multipolar neurons. Two broadly distinct populations of multipolar cells exist: T-stellate and D-stellate neurons, thought to project to inferior colliculus and contralateral cochlear nucleus, respectively. It is unclear which of these neurons receive direct MOC collateral input due to conflicting results between in vivo and in vitro studies. This study used anatomical techniques to identify which multipolar cell population receives synaptic innervation from MOC collaterals. The retrograde tracer Fluorogold was injected into inferior colliculus or cochlear nucleus to label T-stellate and D-stellate neurons, respectively. Axonal branches of MOC neurons were labelled by biocytin injections at the floor of the fourth ventricle. Fluorogold injections resulted in labelled cochlear nucleus multipolar neurons. Biocytin abundantly labelled MOC collaterals which entered cochlear nucleus. Microscopic analysis revealed that MOC collaterals made some putative synaptic contacts with the retrogradely labelled neurons but many more putative contacts were observed on unidentified neural targets. This suggest that both T- and D-stellate neurons receive synaptic innervation from the MOC collaterals on their somata and proximal dendrites. The prevalence of these contacts cannot be stated with certainty because of technical limitations, but the possibility exists that the collaterals may also make contacts with neurons not projecting to inferior colliculus or the contralateral cochlear nucleus.

Keywords: superior olivary complex, olivocochlear neurons, centrifugal pathways, axon collateral, cochlear nucleus. RRID:AB_2336827
Introduction

Sensory systems comprise afferent pathways that send information from the periphery to the brain, and efferent, or centrifugal pathways that enable the brain to influence sensory processing at many levels. In the auditory system of mammals, including humans, the centrifugal control system known as the medial olivocochlear (MOC) pathway, influences early stages of auditory processing (Robertson, 2009; Warr & Guinan, 1979). This projection arises from cholinergic neurons in the ventral and rostral parts of the superior olivary complex in the brainstem and synapses onto the outer hair cells in the cochlea (Brown, 1987; Guinan, Warr, & Norris, 1983; Robertson & Gummer, 1985; Vetter & Mugnaini, 1992; White & Warr, 1983; Winter, Robertson, & Cole, 1989) and activation of the MOC system is known to suppress cochlear neural responses to low level acoustic stimuli (Desmedt, 1962; Rajan, 1988; Wiederhold, 1970; Wiederhold & Kiang, 1970). It has been proposed that this descending system enhances the detection of speech and other signals in noisy environments (Winslow & Sachs, 1987, 1988), a notion supported by behavioural and electrophysiological data (Dewson, 1967; Giraud et al., 1997; Hienz, Stiles, & May, 1998; May, Budelis, & Niparko, 2004; May & McQuone, 1995; May & Sachs, 1992; Mulders, Selukalumaran, & Robertson, 2008; Scharf, Magnan, & Chays, 1997).

MOC neurons also give off collateral branches to the cochlear nucleus, in particular the ventral subdivision (Baashar, Robertson, & Mulders, 2015; Brown, Liberman, Benson, & Ryugo, 1988). Some studies suggest a role for the MOC collaterals in enhancing the ability to respond to signals of interest in the presence of background noise, in concert with peripheral effects of the system (May, Prosen, Weiss, & Vetter, 2002). In addition, we have shown that the responses of certain neurons in the auditory brainstem, recorded in a noisy background, can be restored to the level of the response in quiet when the MOC axons are stimulated (Mulders et al., 2008; Selukumaran, Mulders, & Robertson, 2008b). This represents an additional action to the known anti-masking effect of the peripheral terminations (Winslow & Sachs, 1987, 1988). Furthermore, we showed that excitatory effects observed in CN with stimulation of the MOC axons were still observed after elimination of any peripheral effect (Mulders, Winter, & Robertson, 2002).

Electron-microscopy has demonstrated that the MOC collaterals make synaptic contacts with dendrites of multipolar cells in the cochlear nucleus (T. E. Benson, Berglund, & Brown, 1996; T. E. Benson & Brown, 1990; Brown et al., 1988). However, there are functionally
distinct multipolar neuron types in the ventral cochlear nucleus and it is currently unknown which subpopulation is targeted by the MOC collateral targets. Hence the functional consequences of collateral action for auditory processing remain unresolved.

The multipolar neurons consist of at least two distinct neuronal types, onset chopper neurons and chopper neurons. Onset chopper neurons are known to respond strongly to broadband noise stimuli (Arnott, Wallace, Shackleton, & Palmer, 2004; Mulders et al., 2008; Palmer, Jiang, & Marshall, 1996; Palmer & Winter, 1996; Smith & Rhode, 1989; Winter & Palmer, 1995) and are believed to provide glycinergic inhibitory input to ipsilateral and contralateral cochlear nucleus circuitry (Doucet, Ross, Gillespie, & Ryugo, 1999; Ferragamo, Golding, & Oertel, 1998; Needham & Paolini, 2003; Wenthold, 1987). Onset chopper neurons have been hypothesized to play a crucial role as wideband inhibitory neurons in neuronal networks involved in detection of spectral cues for sound localization (Arnott et al., 2004; Nelken & Young, 1994) and detection of complex signals in noise (Neuert, Verhey, & Winter, 2004; Verhey, Pressnitzer, & Winter, 2003). The second type of multipolar neuron is the chopper neurons. Chopper neurons are narrowly tuned and are thought to provide excitatory input directly to the inferior colliculus (Oliver, 1987; Palmer et al., 1996; Smith & Rhode, 1989). Chopper neurons are thought to be involved in the encoding of complex features of a sound stimulus, essential for the recognition of natural sounds (Wiegrebe & Meddis, 2004; Wiegrebe & Winter, 2001).

We have previously obtained evidence, using a combination of single neuron recordings in the cochlear nucleus in vivo and electrical stimulation of the olivocochlear axons, which suggests that olivocochlear collaterals provide direct excitatory input to the onset chopper neurons (Mulders, Harvey, & Robertson, 2007; Mulders, Paolini, Needham, & Robertson, 2003, 2009; Mulders et al., 2002). However, our data are seemingly at variance with results obtained in slices of mouse cochlear nucleus (Fujino & Oertel, 2001). This latter study investigated the effects of cholinergic agonists (acetylcholine being the neurotransmitter released by M terminals) and showed that D-stellate neurons, which morphologically resemble onset chopper neurons (Arnott et al., 2004; Oertel, Wu, Garb, & Dizack, 1990; Smith, Massie, & Joris, 2005; Smith & Rhode, 1989), were unresponsive to cholinergic agonists. Instead, cholinergic responses were observed in T-stellate neurons, thought to correspond to chopper neurons. Therefore, Fujino & Oertel (2001) suggested that olivocochlear collaterals made excitatory cholinergic connections with T-stellate (chopper) neurons and not with D-stellate (onset chopper) neurons.
Because of the different functional roles of onset chopper and chopper neurons in auditory processing, it is important to establish whether MOC collaterals make synaptic connections with either or both cochlear nucleus subtypes. We therefore used anatomical techniques to investigate the relationship between the MOC collaterals and the different multipolar cells in ventral cochlear nucleus based on their different projection patterns in the auditory system.
Methods

Animals

For this study 41 adult male Wistar outbred rats were used, weighing 280 to 370 grams obtained from the Animal Resource Centre (Murdoch, Western Australia). In addition, 24 outbred, tricolour guinea pigs were used of either sex weighing between 250 to 450 grams obtained from a breeding colony at the University of Western Australia. Experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Ethics Committee of the University of Western Australia. All animals first received a retrograde tracer injection with Fluorogold (FG; Fluorochrome, Denver, Colorado, USA) in either the central nucleus of the inferior colliculus (CNIC) or cochlear nucleus (CN) (details below) followed 5 to 7 days later by an injection with biocytin at the floor of the fourth ventricle. Details on biocytin labelling in rats have been published in a previous paper from our laboratory (Baashar et al., 2015).

Anaesthesia for FG injections

Guinea pigs received a subcutaneous (s.c.) injection of 0.1 ml atropine (Atropine, atropine sulphate 0.6mg/ml, Apex Laboratories, Somersby, Australia) and an intraperitoneal (i.p.) injection of 5 mg/ml Diazepam (Pamlin, diazepam 5 mg/ml, Ceva Animal Health, Glenorie, Australia), followed 20 minutes later by an intramuscular (i.m.) injection of 1 ml/kg of Hypnorm (Hypnorm, 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, VetaPharma, Leeds, UK). Rats were anaesthetised with a single i.p. injection of 60mg/kg Pentobarbitone (Pentobarbitone, pentobarbitone sodium 60 mg/ml, Troy Laboratories, Glendenning, Australia). In both guinea pigs and rats, depth of anaesthesia was confirmed by absence of foot withdrawal reflex. 0.1ml Lignocaine (Lignocaine20, 20mg/ml lignocaine HCl, Troy Laboratories, Glendenning, Australia) was injected s.c. at the site of incision. If foot withdrawal returned during surgery, 0.1ml of Hypnorm was given to guinea pigs and a quarter dose of Pentobarbitone to rats.

FG injections

Once full depth of anaesthesia was achieved, animals were rested on a heating pad and covered with a blanket to maintain body temperature. Animals’ heads were mounted in a stereotaxic frame and the skull exposed. The head was levelled based on lambda and bregma, and the interaural line was used as the anterior-posterior reference point. In both species, the
CNIC and VCN were located using stereotaxic coordinates (Paxinos & Watson, 1982; Rapisarda & Bacchelli, 1977). In addition, in guinea pigs only, correct location was confirmed by recording neural gross responses to sound (see below). A small craniotomy was performed to expose the brain and the bone fragment was retained. Meninges were pierced with a fine hypodermic needle.

Injection pipettes (GC 120-15, Clark Electromechanical Instrument) were pulled using a P-87 micropipette puller (Sutter Instrument Co.) and shortened manually, using fine ophthalmic scissors creating an angled tip under visual control with a microscope (Zeiss West Germany) at 3.2x magnification, to obtain a diameter ranging from 100-120µm. Pipettes were filled with FG solution (Fluorogold, Fluorochrome LLC, U.S.A.; 8% dissolved in 0.9% sodium chloride solution) and connected to a microinjection apparatus (BAB-200, Kation Scientific).

In guinea pigs, pipette placement was confirmed by measuring cluster responses to sound. A silver wire was placed in the injection pipette and reference and earth silver wires were placed in the neck muscles. The recorded signal was amplified (x1000; bioamplifier 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). Sound stimuli were delivered by means of a closed sound system monaurally to the left ear 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 50 ms 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 custom-made program (Neurosound, MI Lloyd).

Two or three iontophoretic injections of FG (10-13µA, 5 second positive pulse on/off cycle for 10 minutes) were made in each animal in either CNIC or VCN in order to maximise the number of retrogradely labelled cells. Following the final injection, the electrode was left in place for 5 minutes before slow withdrawal. The bone fragment was then replaced, scalp sutured, and the animals were rested on a heating pad until full recovery.

Anaesthesis for biocytin injections

Five to 7 days after FG injection guinea pigs were injected s.c. with 0.1ml atropine 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 s.c. To maintain deep surgical
anaesthesia, 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 if foot withdrawal returned. EEG was monitored throughout surgery. In guinea pigs only, when surgical anaesthesia was obtained as determined by absence of the foot withdrawal reflex, a tracheostomy was performed and animals were artificially ventilated on carbogen (95% oxygen and 5% carbon dioxide).

**Biocytin injections**

Details of biocytin injection procedures have been published in detail previously (Baashar et al., 2015). In short, animals were mounted in a stereotaxic apparatus and a large craniotomy (3mm x 4mm) was made to expose the caudal aspect of cortex and cerebellum. In guinea pigs, the cerebellum was partially aspirated to visualise the midline of the floor of the fourth ventricle. The MOC axons were then located using a technique described by our laboratory (Seluakumaran, Mulders, & Robertson, 2008a). Stimulating electrodes (platinum-iridium concentric bipolar electrodes; tip diameter 3 - 4µm, World Precision Instruments, USA) connected to an isolated stimulator output (A-M System, Model-2100) were used to map the thresholds for facial nerve activation at the midline of the floor of the fourth ventricle assessed by whisker twitch (single shocks; 0.1ms duration; rate of 1/s).

Biocytin was then injected at the point of the lowest threshold for evoking whisker twitch. For this purpose, glass micro pipettes (tip diameter 200-230µm) were filled with biocytin hydrochloride solution (Sigma, U.S.A.; 1% in 150mM KCl). Biocytin was then iontophoretically injected (10µA-14µA positive current, 5 second pulse on/off cycle for 3 hours (BAB-200, Kation Scientific)).

In rats, the site for biocytin injections at the floor of the fourth ventricle was determined using stereotaxic coordinates (Paxinos & Watson, 1982). Biocytin was injected as described for guinea pigs above but for a duration of 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).

**Histological processing**

At the end of the biocytin injection period, animals were euthanized with an i.p. injection of 0.3ml Lethabarb (pentobarbitone sodium 325 mg/ml, Virbac Animal Health, Australia) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M phosphate.
buffer (PB, pH7.4). Brains were dissected from the skull and post-fixed in the same fixative overnight at 4°C. The next day, brains were cryoprotected overnight at 4°C in 30% sucrose solution in PB. Transverse brain sections were cut (60µm) using a freezing microtome (Kryomat 1703, Leitz) and collected in PB.

Every 7th section was used for counterstaining with toluidine blue. All other sections were stained free-floating for biocytin at room temperature and sections were rinsed 3x 10 minutes in PB between every step (Baashar et al., 2015). Sections were left 10 minutes in 3% hydrogen peroxide (H₂O₂) in methanol, followed by incubation in 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 in PB for 60 minutes. Then sections were then incubated in ABC solution for 2 hours (1:800, Elite Pk-6100 Standard Vectastain ABC Kit, Vector Laboratories, RRID:AB_2336827) and then reacted for 6 minutes in 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.

**Histological analysis**

For analysis, fluorescence microscopy was used to identify the FG labelled cells and conventional light microscopy to identify the labelled MOC neurons and axons. This method was selected as immunocytochemical detection of Fluorogold proved not strong enough to obtain clear labelling of dendrites. In all successfully double labelled animals (i.e. in which FG injection resulted in multipolar labelled cells in CN and biocytin injection resulted in labelled collaterals in CN) the FG labelled cells in VCN were counted and photographed using a fluorescence 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). Numbers of biocytin labelled cells in SOC and labelled varicosities in VCN were counted using a light microscope with either a 20x objective or a 40x oil-immersion objective.

VCN multipolar cells were defined as cells showing three or more dendrites extending from the soma in different directions. No bipolar or unipolar cells were examined in detail because it could not be excluded that these were multipolar with poor labelling of dendrites. Counting was performed in all biocytin stained sections (6 out of every 7 sections) and therefore neuron counts were corrected for the fact that only 6 out of 7 sections were counted and for double-counting using the Abercrombie correction.
The sizes of FG labelled somata in the CN and biocytin labelled cells in SOC were estimated using NIS Elements Advanced Research software (Nikon version 3) by manually tracing the soma profiles excluding the dendrites at the point of their emergence.

Close appositions between FG labelled profiles and biocytin labelled varicosities were counted as follows. Firstly sections were investigated manually for the existence of a labelled varicosity in close contact with a FG labelled cell body or dendrite 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 with 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 there was possible close apposition of the FG labelled profile and biocytin-labelled varicosity.

Putative close appositions were counted only if the FG labelled profile and biocytin labelled varicosities were both in sharp focus in the same slice of the z-stack. Figure 1 shows two examples where this was found to be the case (Guinea pig: left column and rat: right column). Figure 1 a and b show the merged image of all z-slices indicating a possible contact. Figures 1 c-f are photomicrographs of individual z-stack slices showing the FG labelled profile and biocytin labelled varicosities in focus together (Fig. 1c,d) and a level where they are not (Fig. 1e,f). Figure 2 (Guinea pig: left column and rat: right column) shows two examples of merged images (Fig. 2a,b) that show a possible synaptic contact but close inspection of the z-stack slices showed that the FG labelled profile and biocytin labelled varicosities were never in focus in the same plane (Fig. 2c-f).
Results

**Relationship between MOC varicosities and FG labelled cells**

**Inferior colliculus injections**

Combined anterograde and retrograde labelling experiments from the floor of the fourth ventricle and the CNIC, respectively, were obtained from 4 guinea pigs and 5 rats. Table 1 details the number of FG labelled multipolar cells and biocytin-labelled MOC axonal varicosities in the VCN in these animals. Detailed analysis of the material (see Methods) showed that putative synaptic contacts between the multipolar cells and MOC axonal varicosities in the VCN were extremely rare (Table 1 and see figures 1 and 2 for examples). In both species, less than 0.3% of FG-labelled neurons were found in close apposition to a biocytin-labelled MOC varicosity.

The majority of close contacts of the axonal varicosities observed were with somata of the retrogradely labelled cells (Table 1). Examples of putative synaptic contacts between multipolar cells and MOC axonal varicosities in the VCN contralateral to the FG injection site in CNIC are shown in Figures 1 and 2. In guinea pigs, a higher number of putative synaptic contacts (14/21) was found in the core of the VCN, compared to rats where most putative synapses (13/15) were found on FG-labelled cells near the medial edge of the VCN, particularly in the PVCN. This could be because the MOC axonal varicosities in guinea pigs were located primarily in the core of the VCN, whereas in rats they were located mainly in the medial edge of PVCN and AVCN, as we have previously described (Baashar et al., 2015). In none of the double-labelled animals were any putative synaptic contacts observed in the VCN ipsilateral to the FG injection site. However, bilaterally, in both species many MOC axonal varicosities were observed to make possible synaptic contacts with VCN structures that were not labelled with FG (figure 3).

**Cochlear nucleus injections**

Double-labelling experiments with FG in VCN and biocytin at the floor of the fourth ventricle, were obtained from 3 guinea pigs and 7 rats. The numbers of retrogradely labelled multipolar cells and anterogradely labelled MOC axonal varicosities in the VCN contralateral to the FG injection site in these animals are shown in Table 2. Putative synaptic contacts between the FG labelled multipolar cells and biocytin-labelled MOC axonal varicosities were rare (Table 2). In the 3 double-labelled guinea pigs, no putative synaptic contacts were seen.
In the 7 double-labelled rats, putative synaptic contacts were seen only in two animals, and only these are described below. In the first rat, 2 multipolar cells (1.03% of all FG labelled cells) were observed to make putative synaptic contacts and in the other animal 3 multipolar cells (3.4%). 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. In one rat multiple putative synapses were seen on the proximal or distal dendrites of two FG labelled multipolar cells. This is in contrast to the other rat in which MOC axonal varicosities appeared to make contact only once with a labelled multipolar cell. The majority of putative synapses (13/14) in the rat were found on cells located near the medial edge of the VCN, with only one contact found on a cell located in the VCN core. However, these results must be considered in view of the fact that most MOC axonal varicosities in rats are located in the medial edge.

Similar to what was observed in the double-labelled animals after IC injection, many MOC axonal varicosities were seen to make contact with VCN structures that were not labelled by the FG injections (Figure 3).

**Biocytin labelling**

Biocytin injections were deemed to be successful when labelling was restricted to the MOC axons, no retrogradely labelled neuronal cell bodies were observed in DCN or VCN, labelled neurons were observed in the VNTB and/or RPO and collateral branches could be traced into CN. Unsuccessful injections were placed at the midline but they were more dorsal, caudal or ventral to the facial genua. 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 (Adams & Warr, 1976; Fernandez, 1970). It is worth noting that the caudal and ventral injections did not result in labelling of multipolar cells in the PVCN and AVCN, indicating that the axons of multipolar cells do not leave the VCN via the dorsal acoustic stria. These unsuccessfully labelled animals will not be described further in this section and the following description is restricted to the results obtained from successful injections.

Injections of biocytin resulted in labelling of MOC axons and collaterals in 10 guinea pigs and 14 rats (approximately in 35% of animals). Details of the biocytin labelling in 8 of these rats have been described previously (Baashar et al., 2015) and as the pattern of labelling in all rats was very similar hence detailed description here is limited to the guinea pig results. In the 10 guinea pigs, the apparent size of the biocytin injections varied from 225-607µm.
(384±138) in dorso-ventral aspect, from 154-430µm (286±106) medio-laterally and from 360-840µm (600±164) rostro-caudally. In the 14 rats, the apparent size of the biocytin injections ranged from 175-679µm (369±144) dorso-ventrally, 168-399µm (269±55) medio-laterally and 360-600µm (420±70) rostro-caudally.

In the SOC of guinea pigs, retrogradely labelled cells were distributed bilaterally throughout the medio-lateral and rostro-caudal regions of the VNTB, RPO and DMPO nuclei, while in rats labelled cells were found in the VNTB and RPO nuclei (Figure 4 and see (Baashar et al., 2015)). No biocytin-labelled cells were evident within or around the margin of the LSO in either species (Fig 4d). The cell bodies of the labelled neurons typically showed several dendrites which radiated in different directions from the cell body. Based on their morphological features, these cells were categorized as multipolar cells and their shape and patterns of labelling are consistent with previously described retrograde labelling of MOC neurons (Fig. 4e-g) (Aschoff & Ostwald, 1987, 1988; Robertson, 1985; Robertson, Cole, & Corbett, 1987; Vetter & Mugnaini, 1992; White & Warr, 1983). Numbers of retrogradely biocytin labelled cells varied substantially between animals. The total number of bilaterally labelled cells in guinea pigs ranged from 93 to 424 neurons (263±36) and in rats from 13-1241 (289±84) (see Tables 1 and 2).

Patterns of biocytin labelling of axons and collaterals were similar in guinea pig and rat and confirmed our previous published data (Baashar et al., 2015). Labelled axons were shown to emanate from the rostro-caudal extent of SOC 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 (Fig. 5). The axons were found to course laterally beneath the vestibular nuclei, follow the vestibular nerve root and leave the brainstem. Collateral branches were observed entering the medial edge of the VCN. These collateral branches then ramified, entered the VCN and terminated in various regions of the PVCN and AVCN (Fig. 5). In both species, slightly more collateral branches of MOC axons were observed in the core of AVCN than PVCN. Collateral branches were also observed in the granule cell layer separating the dorsal from the ventral subdivisions of the CN (Fig. 5d) and generally at the medial extent of the granule cell layer. In both species, MOC axons also gave off collateral branches to the vestibular nuclei and the DCN (Fig. 5e,f).

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 the collaterals i.e. en passant and

This article is protected by copyright. All rights reserved.
terminal varicosities, respectively (Fig. 5). These varicosities were stained darkly and uniformly which made them easy to identify. Tables 1 and 2 show the number of the *en passant* and terminal varicosities in the VCN on both sides of both guinea pigs and rats.

**FG Injection Sites**

In each animal, two (CNIC) or three (CN) FG injections were made in an attempt to label a greater number of cells than might be obtained with a single injection. These injections commonly merged together resulting in large injection sites covering large parts of the IC or VCN. Injection sites were characterized by a small necrotic core, surrounded by an area of densely FG labelled cell bodies and a peripheral halo of lightly labelled neuropil.

**Inferior colliculus injections**

Iontophoretic injections of FG into the CNIC were performed in 13 guinea pigs and 25 rats. Though injections spread into the external and dorsal nuclei of the IC, they did not spread into neighbouring structures, such as the contralateral IC, lateral lemniscus or superior colliculus. In guinea pigs, FG injection diameter varied from 384-1257µm (mean = 1002, SD = 207µm) in medio-lateral direction, from 670-1123µm (mean = 894, SD = 122µm) in dorso-ventral direction and from 720-1620µm (mean = 1186, SD = 258µm) in rostro-caudal direction. In rats, the diameter of injection varied from 327-988µm (mean = 593, SD = 208µm) in medio-lateral direction, from 422-1290µm (mean = 745, SD = 312µm) in dorso-ventral direction and from 840-1320µm (mean = 1066, SD = 138µm) in rostro-caudal direction.

**Cochlear nucleus injections**

Injections into the VCN that resulted in contralaterally labelled neurons in CN were made in 11 guinea pigs and 16 rats. The tracer was generally not confined to one subdivision of the CN but typically spread over the posteroverentral CN (PVCN) and anteroventral CN (AVCN), but not into DCN. FG injections did not spread into neighbouring auditory structures such as the SOC or lateral lemniscus. In guinea pigs, the apparent injection size varied from 263-485µm (mean = 321, SD = 61µm) in dorso-ventral direction, from 123-255µm (mean = 213, SD = 48µm) in medio-lateral direction and from 780-1440µm (mean = 1184, SD = 182µm) in rostro-caudal direction. In rats, the apparent size of injection varied from 116-679µm (mean = 395, SD = 193µm) in dorso-ventral direction, from 79-406µm (mean = 231, SD =
109µm) in medio-lateral direction and from 360-1260µm (mean = 814, SD = 249µm) in rostro-caudal direction.

**Distribution of Labelled Cells**

**Inferior colliculus injections**

Distribution of retrogradely labelled cells following FG injections in the CNIC were similar in guinea pigs and rats and hence results are described together. Following injections in the CNIC, labelled cells were found in the contralateral IC, the dorsal, intermediate and ventral nuclei of the lateral lemniscus and some nuclei of the superior olivary complex (SOC), including the lateral superior olive, medial superior olive and dorsomedial part of the olivary complex. Labelled cells were also observed in the dorsal and ventral subdivisions of the CN. No labelled cells were observed in the medial nucleus of the trapezoid body, superior colliculus, trigeminal nuclei, or dorsal column region. These projection patterns are consistent with previous studies in a range of species (Adams, 1979; Beyerl, 1978; Coleman & Clerici, 1987; Kelly, Liscum, van Adel, & Ito, 1998; Kelly, van Adel, & Ito, 2009; Schofield & Cant, 1992, 1999).

Injections in CNIC resulted in labelling of large numbers of neurons 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 6 and 7. The distribution of FG labelled cells over the different subdivisions of the CN was similar in guinea pigs and rats (Figures 4 and 5), though numbers were on average higher in rats than in guinea pigs (Table 1).

In DCN, labelled cells were generally located within the inner layers (layer 2 and layer 3) in agreement with previous reports (Adams, 1979; Brunso-Bechtold, Thompson, & Masterton, 1981; Hackney, Osen, & Kolston, 1990; Oliver, 1984; Webster & Trune, 1982), with fusiform cells being the most common (Figures 6a,b and 7a,b). In VCN, many of the neurons could be characterized as multipolar cells (Figures 6c-f and 7c-g). These observations are comparable with earlier findings in guinea pigs (Hackney, 1987; Pirsig, 1968; Schofield & Cant, 1996b) and rats (Beyerl, 1978; Coleman & Clerici, 1987). Octopus cells and globular bushy cells in the PVCN were never labelled following injections in CNIC and neither were spherical bushy cells in AVCN in agreement with previous data (Beyerl, 1978; Hackney et al., 1990; Harrison & Warr, 1962; Osen, 1969; Webster & Trune, 1982).
**Cochlear nucleus injections**

The spatial distribution pattern of retrogradely labelled cells in the brainstem following FG injections in VCN was similar in guinea pigs and rats. Injections resulted in labelling of large cells in the lateral, ventral and medial nucleus of the trapezoid body, with the most abundant labelling on the ipsilateral side. This is in line with previously reported data (C. G. Benson & Potashner, 1990; Shore, Helfert, Bledsoe, Altschuler, & Godfrey, 1991; Warr & Beck, 1996; Winter et al., 1989).

Retrogradely labelled cells were also observed in the contralateral CN. FG 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 in several species (Cant & Gaston, 1982; Schofield & Cant, 1996a, 1996b; Warr & Beck, 1996; Wenthold, 1987). The retrogradely labelled cells in the DCN were typically found in the deep cell layer, and were rarely present in the outer cell layers. 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 8a and 9a). Based on their morphological features, retrogradely labelled cells in DCN were identified as giant cells, as previously identified in other studies (Cant & Gaston, 1982; Harrison & Irving, 1965; Schofield & Cant, 1996a; Wenthold, 1987).

Labelled cells were widely scattered throughout the rostro-caudal region of the contralateral PVCN and AVCN (Figures 8b and 9b) when the injection site included both subdivisions of the VCN. These findings are similar to those previously described in several species, including guinea pigs (C. G. Benson & Potashner, 1990; Schofield & Cant, 1996b; Shore, Godfrey, Helfert, Altschuler, & Bledsoe, 1992; Wenthold, 1987), rats (Alibardi, 1998a; Doucet, Lenihan, & May, 2009) and cats (Adams & Warr, 1976; Cant & Gaston, 1982).

Retrogradely labelled cells were counted in 11 guinea pigs and 16 rats with good injections in CN. The total number of retrogradely labelled cells in VCN projecting to the contralateral CN ranged from 60-143 in guinea pigs (mean = 91.2, SD = 25.6, n = 11) and from 42-201 (mean = 148.2, SD = 51.4, n = 16) in rats. FG labelled cells were generally most abundant in the rostral PVCN and caudal AVCN, but were also found in the remainder of PVCN and AVCN.

The retrogradely labelled cells in the PVCN and AVCN were categorized as multipolar cells, based on their morphological characteristics and in line with previous reports (Alibardi, 1998a; Cant & Gaston, 1982; Doucet et al., 2009; Schofield & Cant, 1996a, 1996b; Shore et
al., 1992). No other cell types could be identified. Figures 8c,d and 9c,d show examples of large multipolar cells in the PVCN and AVCN of guinea pig and rat, respectively.
Discussion

This study aimed to evaluate the anatomical connectivity of the MOC collaterals in the VCN of rat and guinea pig. The data show that combined retrograde and anterograde tracing can be used to label multipolar cells and MOC collaterals in the VCN. Our results show small numbers of putative synaptic contacts on both types of multipolar cells in line with previous work (Fujino & Oertel, 2001; Mulders et al., 2003, 2009; Mulders et al., 2002). However, the relative density of labelling, even though many MOC axonal terminals and varicosities were labelled, as well as many multipolar cells, was low. Our results suggest that MOC axonal collaterals provide input to the somata and proximal dendrites of CN cells projecting to the contralateral CN and IC, which are presumed to correspond to onset chopper and chopper cells, respectively. However, further studies are required to investigate whether the unlabelled structures in CN in close proximity to the MOC axonal terminals and varicosities represent distal dendrites of CN cells projecting to the contralateral CN and IC or subpopulations of multipolar cells with different projection patterns.

The results and subsequent interpretation are subject to a number of possible technical considerations. Since immunohistochemical staining of FG resulted in only weak labelling of somata and more importantly in very limited visualisation of the dendritic trees this was considered insufficient to allow thorough analysis. Hence this study relied on the fluorescent signal alone for analysis of putative synaptic contacts. While this did result in better revelation of the dendritic arbors, it was subject to fading which may have reduced the number of contacts observed. In addition, we cannot exclude the possibility that contacts are present on distant dendrites that were not easily identified using this tracer technique.

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. It is therefore possible that some of the unlabelled structures in the vicinity of MOC collateral varicosities, could still be cells projecting to contralateral IC or CN.

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 (Guinan, 2006; Vetter & Mugnaini, 1992; Warr, Beck Boche, Ye, & Kim, 2002; Warr, Boche, & Neely, 1997). Therefore, it seems
likely that the results 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 (Brown & Levine, 2008; Robertson et al., 1987; Vetter & Mugnaini, 1992; Warr, 1975, 1980). Thus, injection of the tracer biocytin is expected to result in labelling a large proportion of the axons of MOC neurons. Furthermore, although the number of MOC neurons labelled in guinea pigs was lower than found in some earlier studies using intracochlear injections (Robertson et al., 1987), in some rats the numbers were actually larger than reported by others (White & Warr, 1983).

A final technical consideration is that it cannot be said that close appositions, as described in the results, are true synaptic contacts. This could only be established with certainty using electron microscopy (EM). However, 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. 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 their sheer proximity would suggest (Kasthuri et al., 2015; Ostroff & Zeng, 2015).

On balance, the data do not provide clear support for abundant innervation of the somata and proximal dendrites of cells projecting to contralateral VCN or IC, presumed onset chopper and chopper neurons, respectively, by MOC collaterals in guinea pigs and rats. 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 (T. E. Benson et al., 1996; T. E. Benson & Brown, 1990; Brown & Benson, 1992). Synaptic contacts with excitatory characteristics were described on somata and proximal dendrites of multipolar cells. In addition, these authors 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 (Adams, 1979; Adams & Warr, 1976; C. G. Benson & Potashner, 1990; Beyerl, 1978; Cant & Gaston, 1982; Coleman & Clerici, 1987; Fredrich, Reisch, & Illing, 2009;
Hatano, Ito, Yoshizaki, & Kelly, 2012; Oliver, 1987; Osen, 1972; Schofield & Cant, 1996a; Shore et al., 1992; Shore et al., 1991; Wenthold, 1987).

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 >90% of T-stellate cells, and no effect on D-stellate cells. However, although a lack of effect on the D-stellates is consistent with the present data that suggest limited input to these neurons, the effects they observed on T-stellates are surprising in view of the low innervation observed in this study. 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 be representative of an effect of the MOC collaterals (Sherriff & 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., 2007; Mulders et al., 2003, 2009; Mulders et al., 2002). Using extracellular and intracellular recordings in the VCN, they found that onset-like cells and in particular onset choppers showed excitatory effects following electrical stimulation of MOC axons. After chemical destruction of OHCs by kanamycin or cholinergic blockade of MOC action in the cochlea by strychnine, electrical stimulation of the OCB 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., 2007). These data led to the 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 the data from the electrophysiological in vivo studies from Mulders and colleagues are in conflict with the data obtained by Oertel and colleagues.
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; Schofield & Cant, 1996a, 1996b; Shore et al., 1992; Smith & Rhode, 1989). 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 & Fujino, 2001; Oertel et al., 1990). 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 able to be 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 reports showing 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 (Alibardi, 1998b; Cant, 1981; Smith & Rhode, 1989). 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 (Needham & Paolini, 2003) suggest that some onset choppers do not project to contralateral CN. This is in line with the
anatomical data from Smith et al. (2005) showing 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.

Finally, the present data show 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. In this regard, a recent iontophoretic study from our own laboratory showed a number of cells that responded to the MOCs transmitter acetylcholine that were either unresponsive to sound or could not be classified according to standard criteria (Robertson & Mulders, 2018).

It needs to be borne in mind that 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, 1983; Doucet & Ryugo, 1997; Shore et al., 1992). It should therefore be investigated whether these DCN projecting multipolar cells are in fact the targets of MOC collaterals.
References


This article is protected by copyright. All rights reserved.


Figure legends

Figure 1: Photomicrographs showing two examples of a putative synaptic contact between a FG labelled cell and biocytin labelled axonal varicosity in guinea pig (a,c,e) and rat (b,d,f) in transverse sections of the brain. a,b: Merged images showing a possible contact. c-f: individual z-stack slices showing the FG labelled profile and biocytin labelled varicosities in focus together (c,d) and a level where they are not (e,f). Arrows point at putative contacts. Scale bars: 20 µm.

Figure 2: Photomicrographs showing two examples of seemingly synaptic contacts in transverse sections of the brain between a FG labelled cell and biocytin labelled axonal varicosity in guinea pig (a,c,e) and rat (b,d,f), that proved to be not true close appositions. a,b: Merged image showing a possible contact. c-f: individual z-stack slices showing the FG labelled profile and biocytin labelled varicosities were never in focus in the same plane in focus together. Arrows point at potential contact in the merged image. Scale bars: 50 µm.

Figure 3: Photomicrographs showing examples of putative synaptic contacts between medial olivocochlear axonal varicosities (arrows) and unlabelled VCN structures (stars) in guinea pigs (a and c) and rats (b and d). Scale bars in a-d = 40µm. Transverse plane.

Figure 4: Photomicrographs showing labelled neurons in SOC following successful injections of biocytin at the floor of the IVth ventricle in guinea pig in transverse section of the brain. a: Retrogradely labelled cells in the ventral nucleus of the trapezoid body (VNTB), b: the rostral periolivary nucleus (RPO) and c: the dorsomedial periolivary nucleus (DMPO). d: Absence of labelled cells in the nucleus of lateral superior olive (LSO). e, f and g: High magnification images of medial olivocochlear (MOC) neurons with their extensive dendrites in (e) the VNTB, (f) the RPO and (g) the DMPO. Scale bars in a-d = 100µm, in e-g = 50µm.

Figure 5: 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. Transverse plane, lateral to the left in all panels.

Figure 6: Photomicrographs of typical retrograde labelling in contralateral CN after a unilateral injection of FG into the CNIC in guinea pigs. a: FG labelled cells in DCN. b: High
magnification image of FG labelled fusiform (pyramidal) cell in DCN. c, d: FG labelled cells in the PVCN and AVCN. e, f: High magnification images of FG labelled multipolar cells in the VCN. Scale bars in a = 250µm (also for c and d), in b = 50µm (also for e and f). Transverse plane, dorsal to the top in all panels.

Figure 7: Photomicrographs of typical retrograde labelling in contralateral CN after a unilateral injection of FG into the CNIC in rats. a: FG labelled cells in DCN. b: High magnification image of FG labelled fusiform (pyramidal) cells in the DCN. c, d: FG labelled cells in PVCN and AVCN. e, f, g: High magnification images of FG labelled multipolar cells in VCN. Scale bars in a (also applicable for c and d) = 100µm, in b (also applicable for e, f and g) = 50µm. Transverse plane.

Figure 8: Photomicrographs of retrograde labelling in contralateral CN after a unilateral injection of FG into the VCN in guinea pigs. a, b: FG labelled cells (arrows) in the DCN and VCN. c, d: High magnification images of FG labelled multipolar cells in VCN. Scale bars in a-b = 125µm and c-d = 50µm. Transverse plane.

Figure 9: Photomicrographs of retrograde labelling in contralateral CN after a unilateral injection of FG into the VCN in rats. a, b: FG labelled cells in the DCN and VCN. c, d: High magnification images of FG labelled multipolar cells in VCN. Scale bars in a-b = 200µm and in c-d = 50µm. Transverse plane.
**Table 1:** Numbers of FG labelled neurons (after CNIC injection) and biocytin labelled varicosities in VCN contralateral to the FG injection site and number of MOC labelled neurons in the superior olivary complex (SOC), as well as numbers of putative synaptic contacts and their location in VCN in the double labelled guinea pigs and rats.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>FG labelling</th>
<th>Biocytin labelling</th>
<th>Synaptic targets</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-42</td>
<td>3729</td>
<td>2229</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>GP-41</td>
<td>3518</td>
<td>2283</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>GP-298</td>
<td>1510</td>
<td>1306</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GP-BBE</td>
<td>1097</td>
<td>1629</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-55</td>
<td>5357</td>
<td>1095</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>R-63</td>
<td>4801</td>
<td>410</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R-16</td>
<td>4012</td>
<td>3196</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>R-64</td>
<td>3489</td>
<td>472</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>R-14</td>
<td>2892</td>
<td>1714</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2: Numbers of FG labelled neurons (after CN injection) and biocytin labelled varicosities in VCN contralateral to the FG injection site and number of MOC labelled neurons in the superior olivary complex (SOC) as well as numbers of putative synaptic contacts and their location in VCN in the double labelled guinea pigs and rats.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>FG labelling</th>
<th>Biocytin labelling</th>
<th>Synaptic targets</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Medial edge of VCN</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-75</td>
<td>143</td>
<td>1750</td>
<td>237</td>
<td>0</td>
</tr>
<tr>
<td>GP-76</td>
<td>86</td>
<td>2746</td>
<td>424</td>
<td>0</td>
</tr>
<tr>
<td>GP-78</td>
<td>69</td>
<td>2413</td>
<td>266</td>
<td>0</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-28</td>
<td>201</td>
<td>198</td>
<td>173</td>
<td>0</td>
</tr>
<tr>
<td>R-53</td>
<td>195</td>
<td>1265</td>
<td>338</td>
<td>0</td>
</tr>
<tr>
<td>R-37</td>
<td>194</td>
<td>1504</td>
<td>241</td>
<td>1</td>
</tr>
<tr>
<td>R-40</td>
<td>167</td>
<td>8</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>R-32</td>
<td>164</td>
<td>702</td>
<td>184</td>
<td>0</td>
</tr>
<tr>
<td>R-29</td>
<td>88</td>
<td>1672</td>
<td>402</td>
<td>3</td>
</tr>
<tr>
<td>R-30</td>
<td>42</td>
<td>566</td>
<td>105</td>
<td>0</td>
</tr>
</tbody>
</table>
This study used double labelling techniques to identify multipolar cell populations in cochlear nucleus receiving synaptic innervation from medial olivocochlear collaterals. Our results suggest that both T- and D-stellate neurons receive limited synaptic innervation from these collaterals.