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Research paper

A novel method for selectively labelling olivocochlear collaterals in the rat

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A B S T R A C T

Axons of olivocochlear neurons originate from the brainstem and project to the cochlea. A subpopulation, medial olivocochlear (MOC) neurons, also projects collateral branches to the cochlear nucleus. The precise targets of these collaterals are as yet unknown. Previous methods for labelling these collaterals include firstly, cochlear injections of retrograde tracers, but this is technically demanding and can also label afferent projections or secondly, labelling by injecting tracers into the nuclei of origin of MOC neurons. However, this latter method is non-specific because it also labels non-MOC projections. A technique was used to specifically label MOC collaterals, which involved injections of the tracer biocytin at the floor of the fourth ventricle and fixation 3 hours later. Biocytin injections resulted in labelled neurons in the ventral nucleus of the trapezoid body and rostral periolivary nucleus, confirming MOC axonal labelling. Labelled neurons in dorsal cochlear nucleus indicated labelling of the dorsal acoustic stria and these injections were discarded. After selective MOC labelling, collateral branches were found to innervate granule cell regions, medial edge and core of the ventral cochlear nucleus, as well as the dorsal cochlear nucleus, in agreement with previous data. Therefore we conclude that injections at the floor of the fourth ventricle provide a simple, rapid and specific technique for labelling the majority of MOC axons and their collaterals and this technique may assist in defining the precise neuronal targets of olivocochlear collaterals in cochlear nucleus.

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1. Introduction

The auditory system is composed of ascending and descending pathways. The latter pathways play an important role in enabling the brain to modify afferent auditory input before it reaches the cortex. A component of the descending pathways is the olivocochlear system, which has been divided into two subgroups, the lateral (LOC) and the medial olivocochlear (MOC) system (Guinan et al., 1983; Warr et al., 1979; White and Warr, 1983).

The thin unmyelinated axons of LOC neurons originate from the lateral superior olive (LSO), project exclusively to the ipsilateral cochlea and terminate on the dendrites of the primary afferent neurons beneath the inner hair cells. MOC neurons, on the other hand, originate from the medial, ventral and rostral regions of the superior olivary complex (SOC) and project to the contralateral and ipsilateral cochlea. The thick myelinated axons of MOC neurons travel dorsomedially, form a tight bundle at the midline of the brainstem at the level of the floor of the fourth ventricle and eventually terminate on the outer hair cells (Ginzberg and Morest, 1984; Guinan et al., 1983; Liberman, 1980; Liberman and Brown., 1986; Warr and Guinan, 1979; White et al., 1983). Physiological evidence suggests that the MOC system could play an important role in auditory processing in enhancing the ability to respond to signals of interest in the presence of background noise (Guinan, 2011, 1996, 2006; Kawase et al., 1993; Robertson, 2009; Winslow and Sachs, 1987, 1988). Aberrant behaviour of the MOC system

Abbreviations: AVCN, anterior ventral cochlear nucleus; CN, cochlear nucleus; DCN, dorsal cochlear nucleus; IC, inferior colliculus; IGSB, intraganglionic spiral bundle; LL, lateral lemniscus; LOC, lateral olivocochlear; LSO, lateral superior olive; MOC, medial olivocochlear; OC, olivocochlear; OCB, olivocochlear bundle; PCVN, posterior ventral cochlear nucleus; RPO, rostral periolivary nucleus; SOC, superior olivary complex; VCN, ventral cochlear nucleus; VNTB, ventral nucleus of the trapezoid body

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has also been implicated in tinnitus, hyperacusis and reversible idiopathic hearing loss (Knudson et al., 2014; Rajan, 1989).

Neuroanatomical studies in several species have found that axons of MOC neurons *en route* to the cochlea give off collateral branches to the dorsal (DCN) and ventral cochlear nucleus (VCN) (Benson and Brown, 1990, 1996; Brown, 1993; Brown et al., 1992, 2009, 1991, 1988; Horvath et al., 2000; Ryan et al., 1990; White and Warr, 1983; Winter et al., 1989). The precise function of MOC collaterals in the VCN remains uncertain. As MOC collaterals are thought to be excitatory (Benson and Brown, 1990; Brown and Benson, 1992), it has been proposed that axonal projections of MOC neurons in the VCN may be involved in adjusting central gain, compensating for changes that occur due to the inhibitory action of the MOC system at the auditory periphery (Mulders et al., 2002).

Collateral branches of MOC neurons in the VCN have been labelled in several studies by direct injection of retrograde tracer into the intraganglionic spiral bundle (IGSB) inside the cochlea. The IGSB is the terminal bundle of the OC fibres in the cochlea (Benson et al., 1990, Benson and Brown, 1996; Brown, 1993; Brown and Benson, 1992, 1991, 1988). Injections into this site of the cochlea, however, are technically very difficult, require relatively long survival times for retrograde transport and may label afferent projections as well. In other studies, anterograde axonal tracers have been injected into the nuclei of origin of MOC neurons to label their collateral branches in the VCN. However, such injections resulted not only in labelling of MOC projections in the VCN, but also non-MOC projections originating in the SOC (Warr and Beck, 1996).

Therefore, in this study we introduce a technique that can simply, rapidly and specifically label MOC projections in the VCN. The neuronal tracer biocytin is injected at the centre of the floor of the fourth ventricle where MOC axons are grouped in a tight bundle between the facial genu to label as many as possible of the MOC axons and their axonal branches in the VCN.

2. Materials and methods

2.1. Animal subjects

Experiments were performed on 8 adult Wistar outbred rats of either sex, weighing 280–370 g obtained from the Animal Resource Centre (ARC). 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 study were regulated and approved by the Animal Ethics Committee of the University of Western Australia.

2.2. Anaesthesia

Animals were pre-medicated with a subcutaneous injection of 0.1 ml atropine sulphate (0.6 mg/ml, Apex Laboratories) and anaesthetised with an intraperitoneal injection of 60 mg/kg Pentobarbitone (Pentobarbitone Sodium, 60 mg/ml, Ilium). Depth of anaesthesia was confirmed by absence of foot withdrawal reflex. Once full depth of anaesthesia was attained, the animal's scalp was shaved. A quarter dose of Pentobarbitone was given when foot withdrawal returned.

2.3. Surgical procedures

Once full depth of anaesthesia was achieved, animals were rested on a heating pad to maintain their body temperature throughout the surgery. Heart rate was monitored throughout the procedure by electrocardiogram recording.

The animal's head was placed in a stereotaxic frame (Stoelting Co., 51600). The scalp was incised along the midline. After ensuring the animal's skull was flat and straight, the interaural line was determined to use as the anterior-posterior reference point. The midline suture along the skull was used as the medial-lateral reference point and bregma was used as the dorsal-ventral reference point. An atlas of the rat brain in stereotaxic coordinates was used to determine the site of tracer injections at the floor of the fourth ventricle (1.3 mm posterior to the interaural line, 0 mm to the midline suture, and 8.5 mm ventral to bregma) (Paxinos and Watson, 1982). A dental drill was used to make a small craniotomy in the skull to expose the brain.

Injection pipettes (GC 120-15, Clark Electromechanical Instrument) were pulled using a P-87 micropipette puller (Sutter Instrument Co.) and shortened manually using a fine ophthalmic scissor under visual control with Zeiss West Germany Microscope at magnification 3.2× to obtain a diameter ranging from 200 to 230 μm. Care was taking to ensure that the pipettes were used had angled tips to minimize the chances of blockage. The injection pipettes then were filled with biocytin hydrochloride solution (Biocytin Hydrochloride, Sigma, U.S.A.; 1% in 150 mM KCl), connected to the microinjection apparatus, and lowered to the pre-determined stereotaxic coordinates. Biocytin was iontophoretically delivered by passing positive current between 10 μA and 13 μA, with a 7 s pulse on/off cycle for 80 min (BAB-200, Kation Scientific). After injection, the pipette was left in place for an additional 60 min to allow for sufficient transport of tracer, and then slowly removed.

2.4. Histological processing

After removal of the injection pipette, the animals were euthanized with an intraperitoneal injection of 0.3 ml Lethabarb (Pentobarbitone sodium 325 mg/ml, Virbac animal health, Australia) and perfused transcardially with saline (0.9% sodium chloride) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were dissected from the skull and post-fixed in the same fixative overnight at 4 °C. The next day, the brains were cryoprotected in 30% sucrose solution in 0.1 M PB and left overnight at 4 °C. They were then frozen and sectioned in the transverse plane (60 μm thickness) with a freezing microtome (Kryomat 1703, Leitz). Every 7th section was used for counterstaining with toluidine blue and the other sections were collected for histochemical staining.

Throughout staining, all sections were incubated in glass scintillation vials and agitated on a shaker (Jenk & Kunkel Typ VX7 IKA-VIBRAX-VXR). Sections were rinsed three times in 0.1 M PB and then incubated for 10 min in 3% Hydrogen peroxide (H_2O_2) in methanol to eliminate endogenous peroxidase activity. The brain sections were then rinsed again in 0.1 M PB three times and incubated in 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100 in 0.1 M PB for 60 min at room temperature. Sections were then rinsed three times in 0.1 M PB and incubated at room temperature in ABC solution (1:800, Elite Pk-6100 Standard Vectastain ABC Kit, Vector Laboratories) for two hours. After three rinses with 0.1 M PB, the sections were then reacted for six minutes with DAB solution (0.02% 3,3′-diaminobenzidine (DAB), Sigma) in 0.3% Tris Buffer with 0.2% ammonium nickel sulphate (Univar) and 30 μl of 3% H_2O_2 in 50 ml of double distilled water at a pH 7.6. This procedure resulted in blue-black biocytin labelled cells and fibres. Finally, the sections were rinsed five times in 0.1 M PB.

Reference sections (every 7th section) that were counterstained for identification of cytoarchitectural borders were mounted immediately onto gelatin-coated slides (Menzel-Gläzer) and allowed to air dry overnight. These sections were stained with toluidine blue, dehydrated in graded series of ethanol, cleared in xylene and coverslipped. Sections that had undergone

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