Archives Italiennes de Biologie, 128: 331-353, 1990.

AN EVALUATION OF RETROGRADE TRACING METHODS FOR THE IDENTIFICATION OF CHEMICALLY DISTINCT COCHLEAR EFFERENT NEURONS

D. E. VETTER and E. MUGNAINI

Laboratory of Neuromorphology, Department of Biobehavioral Sciences, Box 11-154, University of Connecticut, Storrs, CT 06269-4154, U.S.A.

INTRODUCTION

Many neuroanatomical studies have sought to visualize the cochlear efferent system by injection of retrogradely transported tracers into the cochlea (3, 4, 5, 6, 8, 11, 19, 36, 42, 43, 44, 45, 48, 51). These studies have shown that there are two main populations of olivocochlear (OC) cells; small neurons are situated lateral to the medial superior olive (MSO) and large neurons are located medial to MSO and are termed the lateral and medial OC systems, respectively. In rats, the small cell group projects ipsilaterally to the cochlea, while the large cell group projects bilaterally. The exact location of the lateral and medial OC neurons varies with the species examined. In particular, small cells are located in the lateral superior olive (LSO) in rats, but lie outside the LSO in cats (48, 51).

Olivocochlear neurons were originally assumed, largely on the basis of their dense staining after histochemical methods for the demonstration of endogenous acetylcholinesterase (AChE), to use acetylcholine (ACh) as a neurotransmitter. Recently, however, a number of studies have indicated that cochlear efferent terminals in the organ of Corti contain many putative neurotransmitters and neuromodulators in addition to ACh (reviewed in 16). Knowledge about the location of chemically distinct cochlear efferent cell bodies and their respective modes of innervation of the organ of Corti, therefore, may be helpful in defining their functional role in audition.

A neurochemical map of OC neurons could be produced by combining neuroanatomical tract tracing with immunocytochemistry, as is done in various other brain regions (reviewed in 20). This combined procedure is carried out by labelling neurons with a retrograde tracer, administered to a territory containing their axon terminal field, and then immunostaining the perikarya with antisera to distinctive endogenous neurochemical markers (10, 20, 22). The visualization of HRP, followed by the PAP immunocytochemical procedure, using two different chromogens such as DAB and heavy metal intensified DAB, is an approach usually preferable to a two step fluorescence protocol since fluophores are not stable. Also, the two non-fluorescent markers can be observed simultaneously in the same cell.

The results of HRP delivery to the cochlea, however, need to be carefully evaluated. Most studies examining the location of OC neurons have employed free

HRP as the retrograde tracer, despite evidence that wheat germ agglutinin conjugated HRP (WGA-HRP) is a much more sensitive tracer than free HRP (18). In addition, the validity of results obtained after cochlear injections of HRP has been challenged by Ross *et al.* (38) who suggested that HRP spreads along the eighth nerve and leaks into the sub-arachnoid space, thus leading to spurious cell labelling in the brainstem. They could not differentiate cells labelled by contaminated CSF from retrogradely labelled, bonafide OC neurons. HRP leakage may also occur through the periotic duct, which connects the perilymphatic space of the cochlea with the sub-arachnoid space. It is known that the duct is susceptible to invasion by small molecules (15) and circulating cells (14, 33) from the sub-arachnoid space. Assuming that there is no directional barrier to flow from the cochlea to the sub-arachnoid space, it is possible that HRP can spread through the duct after cochlear injections, resulting in contaminated CSF and, possibly, spuriously labelled neurons. For instance, Borges *et al.* (9) observed Purkinje cell uptake of numerous tracers injected into the CSF.

This is the first of two related papers. In this paper we present an evaluation of WGA-HRP and free HRP as retrograde tracers for the OC systems and explore which fixative and HRP chromogens are compatible with successive HRP and immunocytochemical staining protocols. In the second paper (47), we utilize the experimental parameters of choice to demonstrate the location of two subsets of OC neurons that may use ACh or GABA as neurotransmitters.

METHODS

Adult Sprague Dawley rats (250-300g) were used for these experiments. The animals were deeply anesthetized with a 3:4 mixture of Rompun (Mobay Corp.) and Ketaset (Bristol-Myers) before surgery (0.10cc/100g i.m.) or Nembutal (Anthony Products Co.) before perfusion fixation (0.15cc/100g body weight, i.p.).

Tracer injections into the cochlea.

In 32 rats the left tympanic bulla was exposed and opened after a post-auricular approach. The stapes was removed from the cochlea and the round window was pierced with an endodontic paper point to slowly remove perilymph. Then a single 3-4μl pulse of either 5% WGA-HRP (Sigma Chem. Co.) in saline (19 animals) or 40% free HRP (Sigma type VI) dissolved in 2% DMSO in saline (henceforth termed 40% HRP, 13 animals) was injected into the scala tympani through the round window using a 29 gauge needle mounted on a microsyringe. To prevent leakage of tracer from the cochlea into the bulla, the round and oval windows were sealed with Histoacryl Blue (Trihawk Int'l., Montreal). The bulla was then swabbed with absorbant endodontic paper points.

After a varying survival period, the animals were perfused transcardially with a saline rinse at room temperature followed by either 800-1000cc of a 0.5% zinc salicylate-formol fixative (27) at room temperature, pH 6.5 or 1000cc of a 0.1M sodium phosphate buffered 1% paraformaldehyde, 1.25% glutaraldehyde fixative at room temperature, pH 7.2. The brains were removed after one hour and either prepared for freeze sectioning by cryoprotection in 30% sucrose in saline at 4° C for two days or immediately sectioned on a Vibratome. All coronal sections (20 μm thick) of the brainstem were serially collected. Alternate sections

were reacted for HRP detection with TMB (24, 25) and cobalt-nickel intensified DAB (2). The TMB reaction product was then stabilized using a two step procedure employing ammonium molybdate tetrahydrate and DAB (20). After the HRP reaction, test sections were processed for immunocytochemistry as specified below. The remaining sections were mounted from gelatin and either counterstained lightly with neutral red (Eastman Kodak Co.) or left uncounterstained. All sections were coverslipped with Permount. The HRP tracing experiments and the controls described below are summarized in Table 1.

Table 1. — *Details of the various experimental parameters used to find the optimal processing paradigm for the demonstration of retrogradely labelled olivocochlear neurons.* Abbreviations: HRP, horseradish peroxidase; WGA-HRP, wheat germ agglutinin conjugated horseradish peroxidase; CM, cisterna magna; ZnAld, zinc salicylate- formol fixative; F/G, paraformaldehyde-glutaraldehyde fixative.

Tracer	*Application Method*	*Survival Time (hrs)*	*Perfusate and number of subjects*
WGA-HRP (5%)	CM Pellet	24	Zn/Ald (1)
			F/G (3)
	Cochlear Injection	15	ZnAld (3)
		18	ZnAld (3)
		21	ZnAld (6)
		24	ZnAld (3)
		48	ZnAld (2)
		96	ZnAld (2)
HRP (40%)	CM Pellet	24	ZnAld (1)
			F/G (1)
		48	ZnAld (1)
			F/G (2)
	Large CM Pellet	48	ZnAld (1)
	CM Injection	24	ZnAld (2)
	Cochlear Injection	24	ZnAld (2)
			F/G (2)
		48	ZnAld (2)
			F/G (2)
		72	ZnAld (1)
		96	ZnAld (1)
			F/G (3)

Control tracer administrations.

Three procedures were performed as controls. Anesthetized rats were mounted in a stereotaxic device. The membrana atlanto-occipitalis was exposed and opened. After CSF flow subsided, a 2mm (nine animals) or 5mm (one animal) cotton pellet, soaked with either 40% HRP or 5% WGA-HRP, was placed in the cisterna magna (CM). The membrane was immediately sealed with a latex square held in place with Histoacryl Blue to prevent

post-operative CSF leakage. The animals survived 24 or 48 hours, after which they were perfused and the brains cryoprotected, cut, and reacted as described above. In two animals, a 33 gauge needle was passed throught the membrana atlanto-occipitalis and 40% HRP (5μl) was injected into the CM using a microsyringe. The CM was then sealed with a latex square held in place with Histoacryl Blue. After 24 hours, the rats were perfused and the brains processed as detailed above. Finally, in one animal the tympanic bulla was exposed with a post-auricular approach. The bulla was then opened, leaving the cochlea and osseous chain intact, and five microliters of HRP were dripped into the cavity. After 48 hours, the animal was perfused with the paraformaldehyde and glutaraldehyde fixative detailed above. The brain was then cryoprotected, and frozen sections were finally reacted with cobalt-niclel intensified DAB for the presence of HRP. The sections were counter-stained and mounted as detailed above.

Immunocytochemistry.

Several of the HRP reacted sections were incubated in an antiserum to either sheep anti-GAD or goat anti-ChAT in order to investigate the effects different tract tracer protocols would have on immunostaining.

GAD immunoreactive neurons were localized using the indirect, peroxidase anti-peroxidase (PAP) technique carried out as a double PAP, double DAB sequence (29). The procedure has been detailed elsewhere (27, 31). Briefly, the sections were incubated for one hour in 1% normal rabbit serum followed immediately by a 48 hour incubation at 4° C in sheep GAD antiserum S3 (30) at a 1:2000 dilution or in normal sheep serum, 1:1000, for controls. Secondary and tertiary incubations employed rabbit anti-sheep IgG (1:50, ICN Immunobiologicals) and goat PAP (1:100, Sternberger Meyer) respectively. DAB was then used as the chromogen. ChAT immunoreactive neurons were localized using a polyclonal goat ChAT antiserum (gift from Dr. L. Hersh) at 1:2000 dilution. Control sections were incubated in normal goat serum diluted 1:1000. Thereafter, the same procedure followed for GAD visualization was employed for ChAT localization. The diluent for all steps and washes was 0.5M Tris HCl, pH 7.6. Control sections did not show any specific immunostaining.

Cochlear efferent cell quantification.

The numbers of HRP labelled cells in sections of the auditory brainstem of two animals were counted 48 hours after free HRP was injected into one cochlea. Also, the distribution of OC neurons in the different LSO limbs was noted. Cells were observed and counted at a final magnification of approximately 400x using an Olympus BH microscope. Only cells with a nucleus were counted. No corrections were made to account for split nuclei. The actual number of cells may be up to 30% smaller than reported here (1, but see also 52). We prefer to show raw data for comparison with White and Warr (51), who used uncorrected cell counts.

Abbreviations

AChE	:	acetylcholinesterase
CM	:	cisterna magna
CN	:	cochlear nuclei
CSF	:	cerebral-spinal fluid
DAB	:	3,3'-diaminobenzidine tetrahydrochloride
DMSO	:	dimethylsulfoxide
HRP	:	horseradish peroxidase

LSO : lateral superior olive
LVPO : lateroventral periolivary region
MSO : medial superior olive
MVPO : medioventral periolivary region
NTB : nucleus of the trapezoid body
OC : olivocochlear
OCB : olivocochlear bundle
RPO : rostral periolivary region
SOC : superior olivary complex
SPN : superior paraolivary nucleus
TMB : 3,3',5,5'-tetramethylbenzidine
VLL : ventral-lateral lemniscus
WGA-HRP: wheat germ agglutinin conjugated horseradish peroxidase.

RESULTS

1. *Tracer injections into the cochlea.* — WGA-HRP: The 19 rats which received cochlear injections (3-4μl) of 5% WGA-HRP were divided into six subgroups based upon survival times of 15, 18, 21, 24, 48, or 96 hours. After survival times of 15-48 hours, all of the animals exhibited two prominent HRP reaction patterns: the ventral surface of the brainstem was stained (as described below), and a granular HRP reaction product was observed in neurons of the superior olivary complex (SOC), the area adjacent to the genu of the facial nerve, and in the ventral subceruleus nucleus. The SOC was parcellated according to Osen *et al.* (32). Labelled neurons could be found bilaterally within the medioventral periolivary region (MVPO) (Fig. 3), rostral periolivary region (RPO) and ventral subceruleus nucleus. Cells in the ipsilateral cochlear nucleus (CN) (Fig. 2B) contained HRP reaction product. Small cells within the lateral superior olive (LSO) and large cells in the lateroventral periolivary region (LVPO) and along the dorsal and medial borders of the medial LSO limb and lateral border of the lateral LSO limb were also labelled ipsilaterally. Most notably, both ipsilateral and contralateral neurons of the nucleus of the trapezoid body (NTB) contained HRP granula (Figs. 1, 4A-C). Labelling of rat NTB and CN neurons from the cochlea had not been reported previously (see discussion).

After 96 hours, the surface staining of the brainstem was limited to the pial border. The number of labelled neuronal cell bodies, however, increased dramatically. Labelled cells appeared in all superficial areas of the brainstem and cerebellum. Many labelled neurons were observed in regions clearly not involved in any known projection to the cochlea, namely the cochlear nuclei, ventral tegmental nuclei, locus ceruleus, raphe nuclei (raphe pallidus in particular), cerebellar cortex, dorsal and external cortices of the inferior colliculus, nuclei of the lateral lemniscus, MSO, and superior paraolivary nucleus (SPN). Additionally, the LSO was bilaterally labelled. Whereas trans-neuronal transport could account for labelling observed in acoustic nuclei, the tracer at the surface of the brainstem was evidently the source of most of this excessive neuronal labelling.

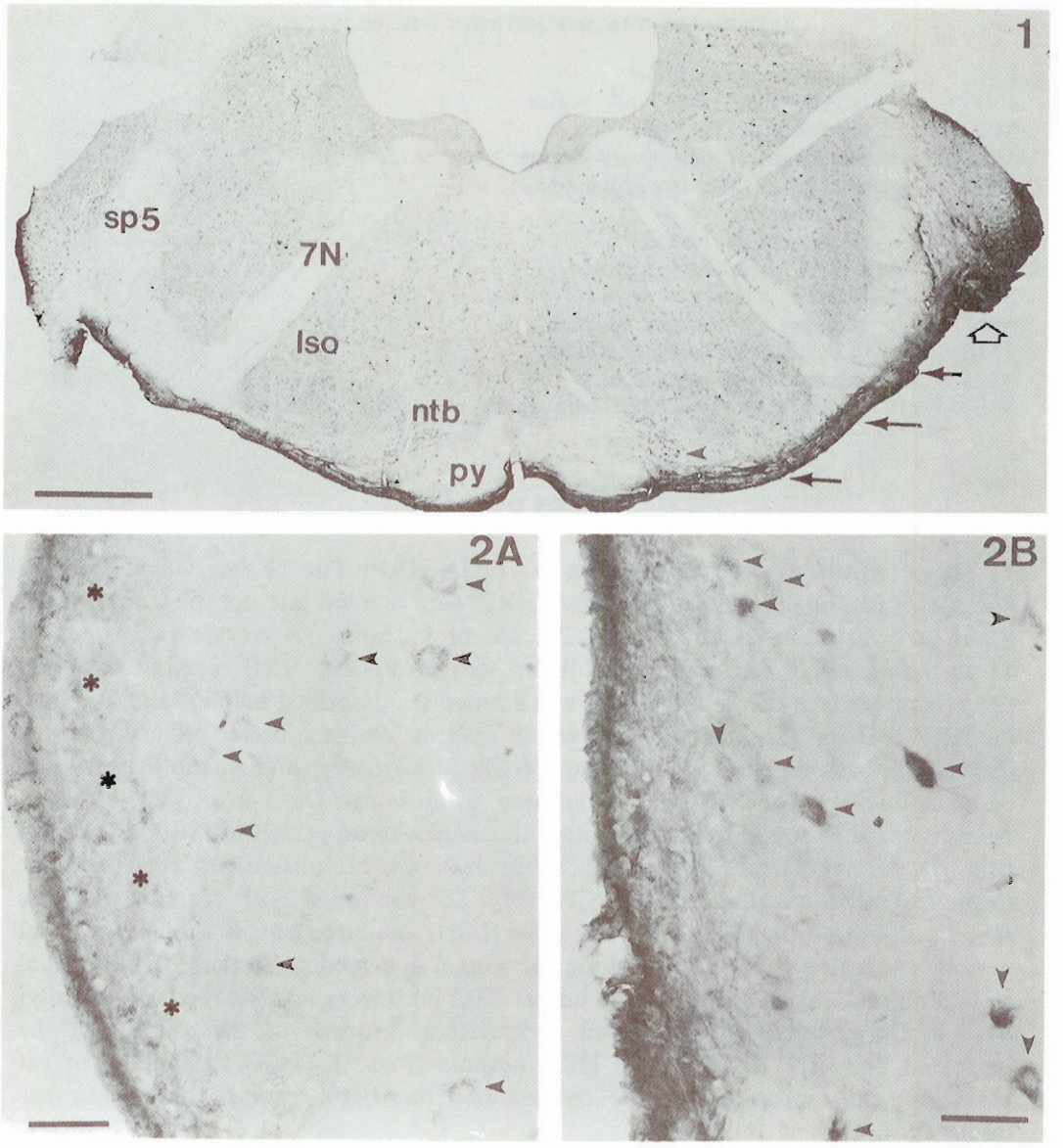


Fig. 1. – *Ventral brainstem rim.*

After injecting 3-4µl of 5% WGA-HRP into the cochlea (ipsilateral to the open arrow), tracer leaks into the CSF and stains the ventral brainstem surface (solid arrows). Also, cells in the vicinity become spuriously labelled (arrowhead). Metal intensified DAB; 21 hrs. survival; counterstained with neutral red; scale bar 1 mm.

Fig. 2A. – *Portion of the CN after a WGA-HRP soaked pellet has been placed into the CM.*

Note light, diffuse rim (asterisks) and labelled cell bodies (arrowheads). Metal intensified DAB; 24 hrs. survival; no counterstain; scale bar 50µm.

Fig. 2B. – *Portion of the CN after WGA-HRP injection into the cochlea.*

Many cells (arrowheads) become labelled as does the surface of the brain. TMB stabilized with DAB; 15 hrs. survival; no counterstain; scale bar 50µm.

Fig. 3. – *Following a WGA-HRP cochlear injection, there is a brainstem rim (delineated by small arrowheads) and cellular labelling (large arrowheads) in the MVPO region.*

Pericytes can be observed (asterisks) due to their endogenous peroxidase. TMB; 15 hrs. survival; no counterstain; scale bar 50μm.

The surface staining of the brainstem deserves an accurate description because of its uncommon occurrence as a complication of tract tracing studies involving injection of solutions into the cochlea. In all animals that received cochlear WGA-HRP injections, the ventral, and at times the lateral, surface of the brainstem exhibited a diffuse staining after both the DAB and TMB procedures (Fig. 1). The staining spread from the surface to varying depths and mediolateral extents of the brainstem depending on the length of survival. At the shortest survival time (15 hours) the ventral surface staining was found under the NTB ipsilateral to the injected cochlea and extended for only a short distance into the trapezoid body (Fig. 4A). Greater depths and mediolateral extents of the trapezoid body region became stained bilaterally after 48 hours (Fig. 4C). Also, there was a correspondence between the intensity of staining of the surface rim and the number and intensity of some of the auditory brainstem nuclei labelled. For example, three animals with limited diffuse staining of the trapezoid body contained labelled cells in NTB which were restricted to the ventral-most portions of the nucleus (Fig. 4A-B), while more extensive diffuse staining led to labelling of the majority of NTB cells, the most ventral of which became stained the most intensely (Fig. 4C). The diffuse brainstem labelling was reminiscent of the results reported by Ross *et al.* (38) and made unequivocal identification of cochlear efferent neurons impossible. As already mentioned, the diffuse surface staining nearly disappeared after 96 hours, but the number of labelled neurons, especially in areas not previous-

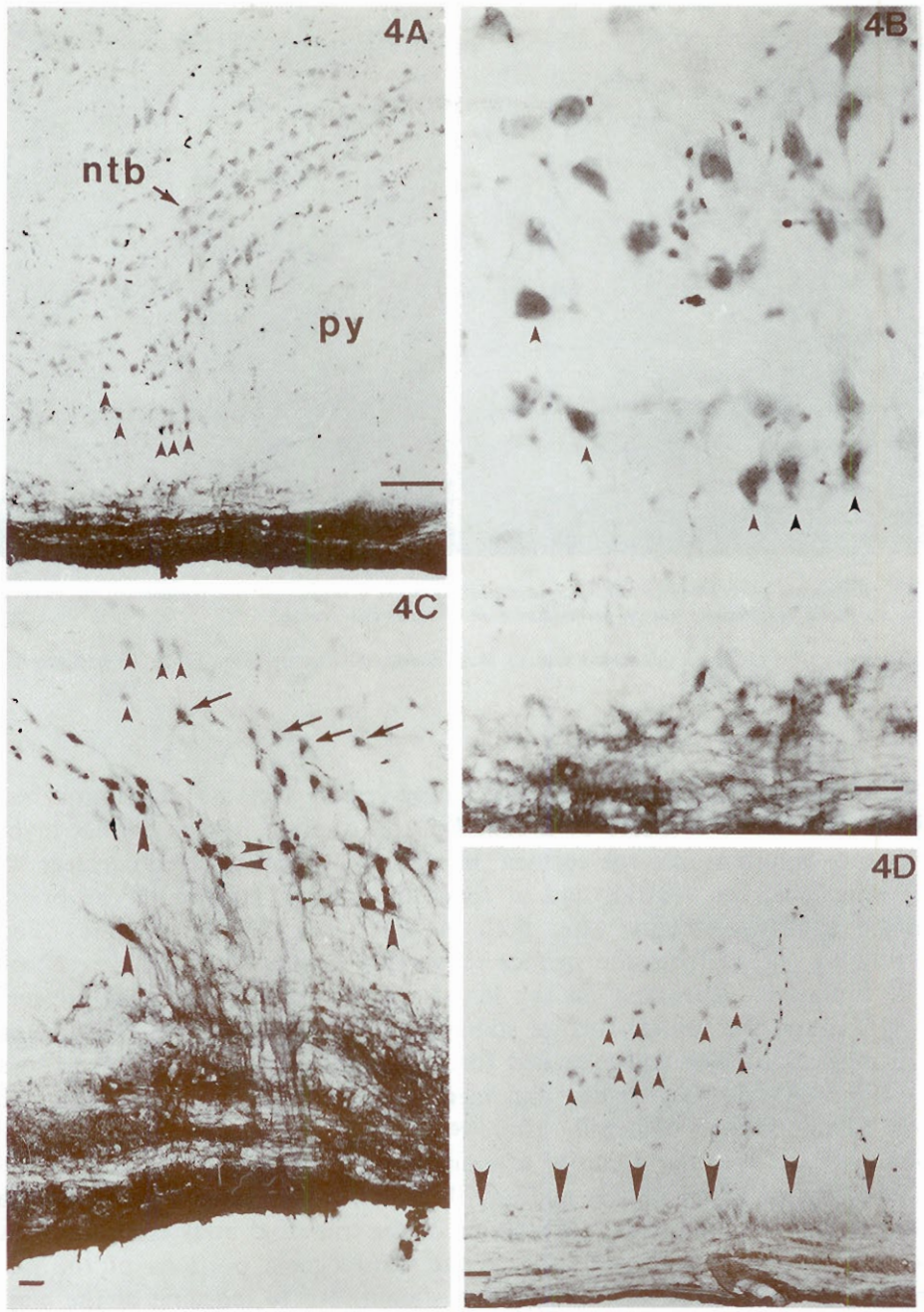


Fig. 4. – *WGA-HRP produces artifactual staining, making it difficult to identify retrogradely labelled cells.*

4A: After even a limited surface rim is produced, some cells (arrowheads) still become labelled in NTB (py, pyramids). Cochlear injection of WGA-HRP; metal intensified DAB; 24 hrs. survival; neutral red counterstain; scale bar 100μm.

Fig. 5. – *Following free HRP cochlear injections, the eighth nerve (white asterisk), primary afferents (white triangle), efferent vestibular fibers (arrows) and olivocochlear fibers (arrowheads) become labelled.*

Perivascular cuffs (black asterisks) sometimes occur, indicating HRP penetration into the brain over blood vessels (ref. 34), but no surface rim or spurious cellular labelling is discernable. Metal intensified DAB; 24 hrs. survival; no counterstain; scale bar 1 mm.

4B: Higher magnification of NTB area in figure 4A. Arrowheads point to same cells in 4A and 4B. Scale bar 20μm.

4C: After a typical surface rim is produced, the majority of NTB cells are labelled. There is a ventral to dorsal gradient of labelling intensity with the most intense cell body staining (large arrowheads) located ventrally and the weakest cell body staining (small arrowheads) occurring dorsally. There is a transition zone of cells which are moderately labelled (arrows). Cochlear injection of WGA-HRP; metal intensified DAB; 15 hrs. survival; no counterstain; scale bar 20 μm.

4D: After placing a WGA-HRP soaked pellet into the CM, a faint surface rim (large arrowheads) is produced and NTB cells (small arrowheads) become labelled indicating that cell bodies may be spuriously labelled with HRP originating from the CSF. Metal intensified DAB; 24 hrs. survival; no counterstain; scale bar 20 μm.

ly reported as containing OC neurons, increased dramatically throughout the brainstem.

FREE HRP: The 13 rats which received 40% HRP in the left cochlea were divided into four subgroups according to survival times of 24, 48, 72, and 96 hours. In these rats there was no evidence of diffuse staining of the ventral brainstem or of labelled neurons within NTB. Therefore, the brain of these animals was studied in greater detail.

Fibers and axonal terminals were observed in the ipsilateral CN (Fig. 5). Also, the OCB and some vestibular fibers were stained. The OCB could be traced to its decussation under the floor of the fourth ventricle. Within the SOC, the LSO and LVPO (ipsilaterally) and the MVPO and RPO (bilaterally) contained labelled neurons as early as 24 hours after cochlear HRP injections, but the number of cells and the amount of HRP granules within the cells increased with greater survival times up to 48 hours. Small spherical cells 13μm in average diameter were located in all limbs of the LSO (Figs. 6 and 8) while large spindle-shaped or rounded cells (18-20μm in long diameter) were situated within and immediately dorsal to the fiber capsule at the dorsal, medial, and lateral aspects of the ipsilateral LSO (Figs. 6 and 7). Cells in the LVPO were spherical and 21μm in average diameter while cells in the MVPO and RPO (Figs. 9 and 10) were similar in size and shape to those located along the borders of the LSO. In some animals, the ipsilateral MVPO and RPO contained a few small (12-13μm in diameter) retrogradely labelled cells. Such cells were ipsilateral to the injected cochlea. The relevance of these cells is examined further by Vetter *et al.* (47). A summary diagram of the location of cochlear efferent neurons in the auditory brainstem is presented as Fig. 11.

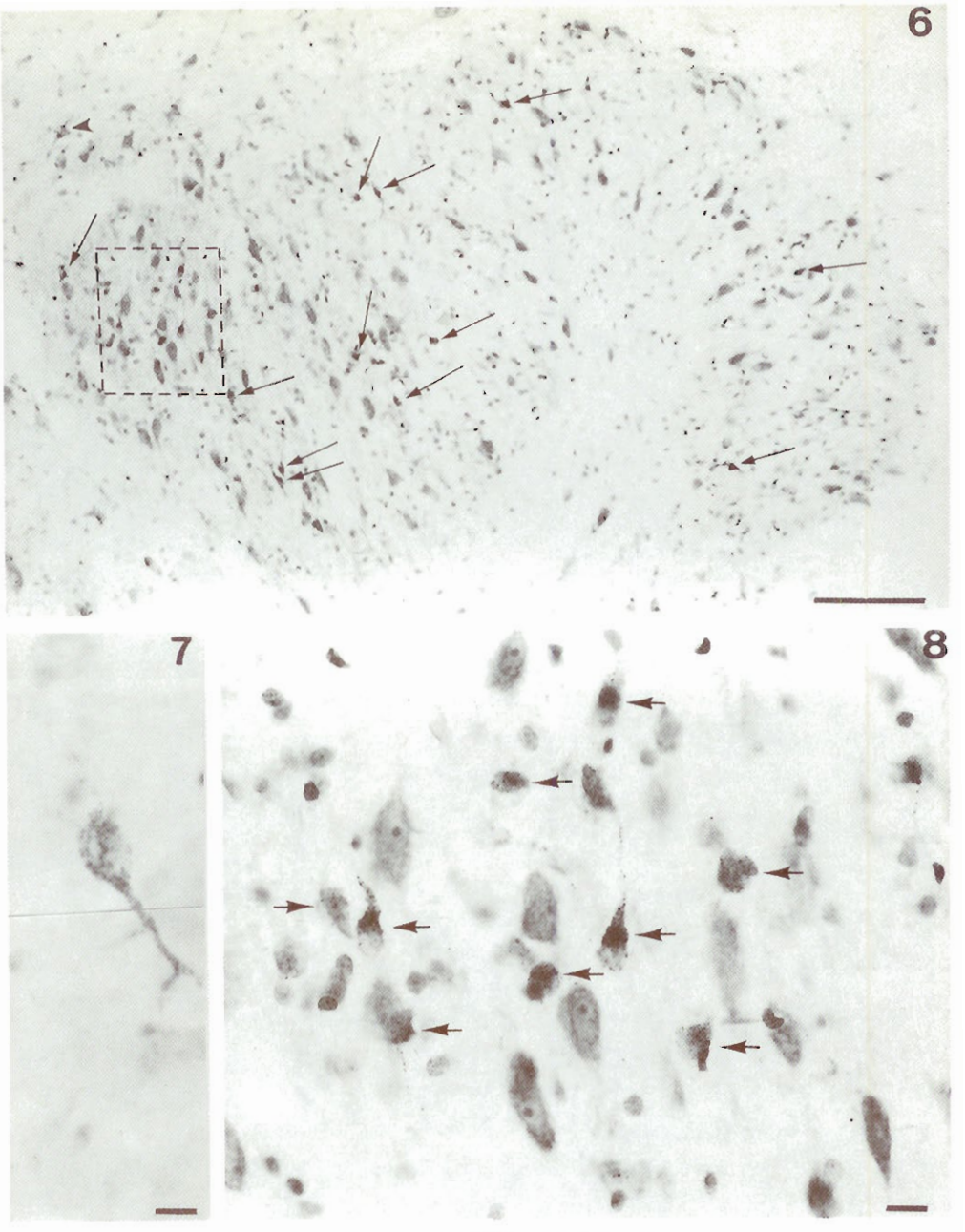
Table 2 contains the number of OC neurons counted in the SOC. The total number of OC neurons in the SOC is only slightly larger than that published by White and Warr (51). When MVPO and RPO cell counts are added together, one obtains the total number of cells in what White and Warr (51) have classified VNTB. White and Warr (51) have estimated that the medial system of rats is composed of cells located contralaterally and ipsilaterally in approximately a 65:35

Table 2. — *The number of cochlear efferent neurons counted in two rats in each of the main SOC nuclei.*

Number of OC Neurons in the rat SOC

	Ipsilateral				*Contralateral*				
animal #	LSO[1]	LVPO	MVPO	RPO	LSO	LVPO	MVPO	RPO	total
4621	339 (24)	4	99	130	0	0	95	71	762
4622	152 (15)	8	92	93	0	0	191	108	659
mean	245 (20)	6	96	111	0	0	143	90	711

[1] The number in parentheses indicates the number of large OC neurons counted which were located within and immediately dorsal to the fiber capsule of the LSO.



Figs. 6-8. – *Cell labelling in SOC after injecting HRP into the cochlea.*

Metal intensified DAB; 48 hrs. survival; neutral red counterstain.

Fig. 6. – Small retrogradely labelled neurons (arrow) are located in the body of LSO and large retrogradely labelled neurons (arrowhead) are around LSO. Scale bar 100μm.

Fig. 7. – Large retrogradely labelled cells are found along the dorsal and medial portions of the medial limb of the LSO and along the lateral side of the lateral LSO limb. Scale bar 10μm.

Fig. 8. – Higher magnification of the boxed area in Fig. 6. Many small retrogradely labelled cells can be discerned within LSO after injecting HRP into the homolateral cochlea. Note that even after using a fixative designed for optimizing immunocytochemistry, HRP granules remain intact and in plentiful supply. Scale bar 10μm.

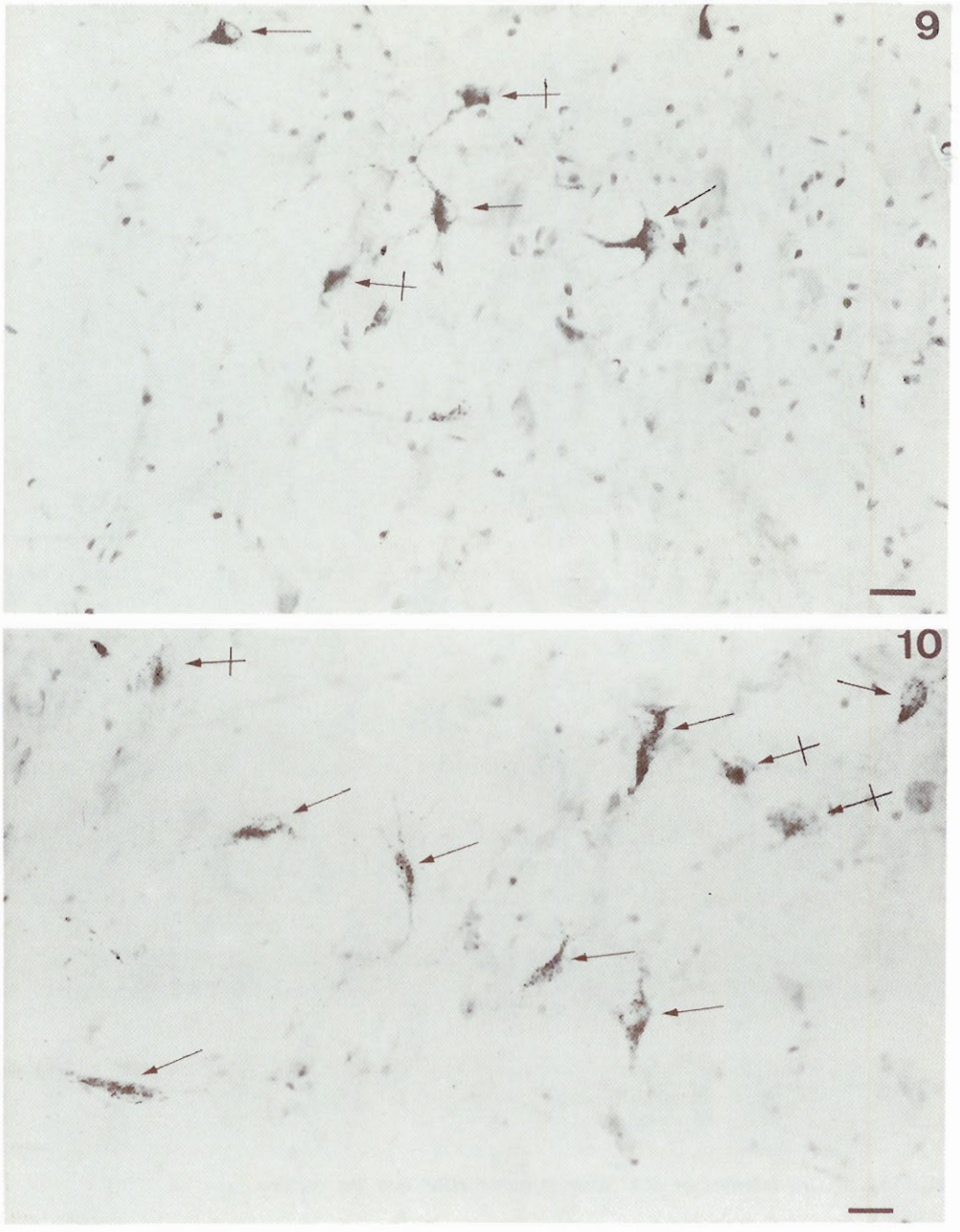


Fig. 9 and 10. – *Cell labelling in SOC after injecting HRP into the cochlea.*

Same methods and survival time as in Figs. 6-8.

Fig. 9. – Large retrogradely labelled cells are located bilaterally in MVPO (arrows). Several cells are out of the plane of focus (crossed arrows). Scale bar 20μm.

Fig. 10. – Large retrogradely labelled cells are found bilaterally in RPO. Scale bar 20μm.

11

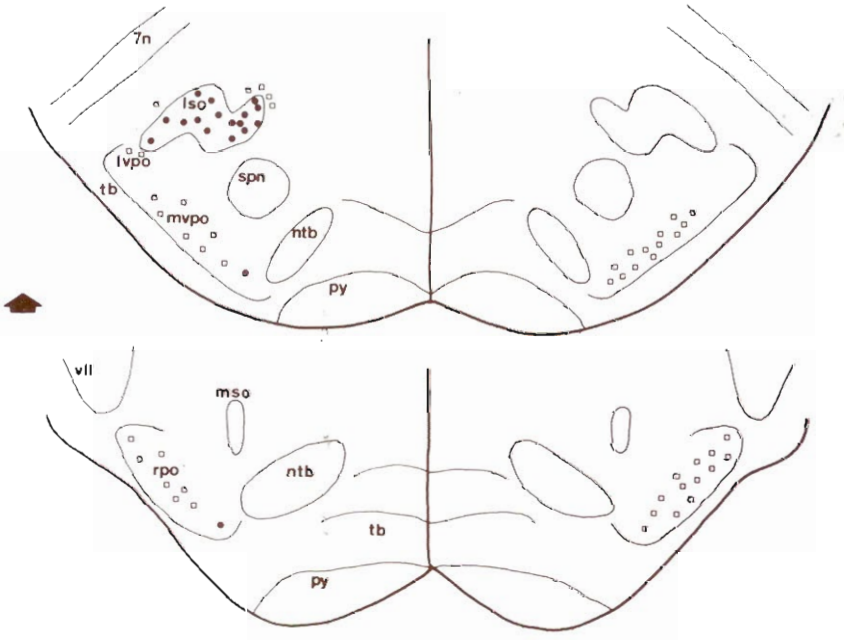


Fig. 11. – *Summary diagram of the location of rat OC neurons after injection of HRP into the cochlea (side indicated by large arrow head).*

In general, small cells (solid circles) are found ipsilaterally in the body of the LSO, but a few rare small cells are found in MVPO and RPO. Within LSO, the majority of cochlear efferent cells are located in the medial LSO limb. Large cells (open squares), on the other hand, are found bilaterally in MVPO and RPO. Approximately two-thirds of the OC cells in MVPO and RPO are located contralateral to the injected cochlea. Large cells surrounding LSO, however, are found only ipsilateral to the injected cochlea. Cochlear efferent cells in LVPO are located ipsilateral to the injected cochlea.

ratio. In animal # 4622, which had the largest total number of medial OC neurons (484), presumably indicating extensive HRP uptake from all areas of the cochlea, our count of 62% contralateral cells and 38% ipsilateral cells agrees closely with the results obtained by these authors.

We investigated the distribution of OC neurons within the rat LSO. The borders between these limbs were arbitrarily traced in coronal sections by extending vertical lines from the apex of each hilus to the opposite curvature of the gray matter. The number of retrogradely labelled cells was counted in each of the three constituent limbs. The medial limb contains approximately 50% of all OC neurons found in the LSO, while the middle and lateral limbs contain 32% and 17% of the retrogradely labelled cells respectively. This distribution has not been previously reported in rats.

A number of areas outside the SOC contained HRP labelling. The ipsilateral

cochlear nucleus (CN) contained only HRP stained terminals at short survival times (Fig. 5), but after 72 hours some CN neurons became stained, presumably due to trans-neuronal labelling. Small neurons distributed bilaterally near the genu of the facial nerve were retrogradely labelled. This area has been identified physiologically as containing vestibular efferent neurons (13, 17) and we therefore conclude that these cells are retrogradely labelled by HRP which invaded the vestibular labyrinth during the cochlear injection. A number of retrogradely labelled cells, some bilaterally disposed, were located in the ventral subceruleus nucleus, which has been shown to contain central preganglionic parasympathetic neurons in rats (12). It should be noted in this respect, that our surgical procedure involves the removal of the stapes, producing damage to the chorda tympani, which runs over portions of the osseous chain. Cells in the subceruleus nucleus are presumably labelled as a result of uptake of HRP by the damaged chorda tympani. They were not labelled after control injections of HRP into the bulla.

Overall, more cells were labelled by WGA-HRP than by HRP. However, the conspicuous diffuse labelling at the brainstem surface obtained after WGA-HRP cochlear injections engendered the problem of determining which cells were retrogradely labelled from the cochlea (OC neurons) and which were labelled due to the spread of the tracer from the brainstem surface. A series of experiments was therefore conducted to further investigate which areas of the brainstem become labelled when the tracer is administered to the surface of the brainstem, i.e. outside the cochlea.

2. *Tracer administration into the cisterna magna.* — A cotton pellet soaked with 5% WGA-HRP was inserted into the CM of four rats, which were then allowed to survive 24 hours. The animals were perfused with either the zinc salicylate-formol or the paraformaldehyde and glutaraldehyde fixative and sections of the auditory brainstem were processed as described earlier. All of the subjects exhibited labelled cells bilaterally within the raphe pallidus, locus ceruleus, cochlear nucleus (CN, Fig. 2A), NTB (Fig. 4D), and the ventral portions of MVPO and LVPO. Also, a diffuse ventral brainstem surface staining was present, but was not as prominent as observed after cochlear injections of WGA-HRP (compare Fig. 2A with 2B and Figs. 4A and 4C with 4D).

A cotton pellet soaked with 40% HRP was inserted into the CM in six animals. Thereafter, two animals survived for 24 hours and four animals survived for 48 hours. The 24 hour survival group showed no evidence of ventral brainstem staining or neuronal uptake of HRP. In the 48 hour survival group, three animals showed no signs of a diffuse brainstem surface staining or of neuronal labelling while one animal, which received a large HRP soaked cotton pellet, exhibited extensive uptake into NTB and MVPO, locus ceruleus, and raphe neurons, along with a faint brainstem surface staining. However, no labelled cells were present in LSO, LVPO, or RPO.

Two animals received needle injections of approximately 5μl of 40% HRP into the CM. Contrary to the results reported by Warr using kittens (48), there was

extensive bilateral uptake of the tracer into all nuclei of the SOC as well as the CN following a 24 hour survival period. However, no diffuse brainstem staining was discernible. Many non-auditory centers were labelled, namely the raphe pallidus and pontis nuclei, the lateral and medial parabrachial nuclei, the principal sensory trigeminal nuclei, the spinal trigeminal nuclei, the medial longitudinal fasciculi, and the facial nerve nuclei. All cells labelled after HRP was injected into the CM contained very small, dust-like HRP reaction product, not the large, more coarse granula observed after retrograde labelling from the cochlea.

3. *Tracer administration into the tympanic bulla.* — Forty-eight hours after 5μl of HRP was placed into the tympanic bulla there was no detectable HRP reaction product in the brain. Since no damage was done to the cochlea or neighboring nerves, this is not surprising. This observation should not be viewed as conflicting with the results obtained by White and Warr (51), since these authors administered HRP into the bulla after opening the facial canal. Such a procedure resulted in retrograde labelling of neurons in the ventral subceruleus nucleus and facial motor nucleus.

4. *Non-neuronal labelling.* — Occasionally, brainstem sections stained with the TMB procedure after application of either tracer to the cochlea or the CM contained a varying amount of fine dispersed granules. These were more frequent near the ventral surface of the brainstem and decreased progressively in more dorsal areas until there were none in or immediately under the LSO. Such granules probably represent WGA-HRP or HRP which has diffused from the CSF into the interstitial fluid and been internalized at low density by cellular elements. Many of these granules are presumably situated in the perikaryon and processes of glial cells, as pointed out by Warr *et al.* (50, their Fig. 13C). Some labelled glial cells could clearly be distinguished under the floor of the fourth ventricle of animals which had undergone the CM procedures. It appeared that WGA-HRP produced greater glial labelling than free HRP. Also, tracer application into the CM resulted in greater glial labelling than did cochlear injections. Furthermore, pericytes were observed within blood vessel walls (Fig. 3) after TMB or DAB reactions, but never presented a problem of interpretation due to their location and intense reaction product, at least part of which may be due to endogenous peroxidase.

5. *Effects of different fixatives and chromogens.* — Two fixatives were selected for this study: 1) a paraformaldehyde and glutaraldehyde fixative optimal for the HRP reaction (25); and 2) a zinc salicylate-formol fixative which has already been shown effective in optimizing immunostaining (27). The paraformaldehyde-glutaraldehyde fixative was superior for the visualization of HRP in cell bodies, but prevented satisfactory immunostaining for GAD and ChAT, two intrinsic markers expressed by different populations of OC neurons (47). The zinc salicylate-formol fixative did not appreciably degrade HRP within cell bodies (Figs. 8 and

9) when the perfusion time was short (one hour total exposure time to the fixative) and the temperature did not exceed 20° C. In addition, this fixative allowed subsequent immunostaining for both GAD and ChAT (for photographic documentation, see 47).

No observable difference occurred in the number and location of retrogradely labelled cells or in the extent of staining at the surface of the brainstem when TMB or cobalt nickel intensified DAB was used as the HRP chromogen. The TMB reaction was more sensitive than the DAB reaction, judging by the amount and intensity of the reaction product within the cell body and processes, in accord with previously published studies (22). The TMB reaction product, however, required stabilization with ammonium molybdate tetrahydrate and DAB (21) prior to immunostaining. Both before and after stabilization, labelled cell bodies, especially small cell bodies, were completely packed with HRP granula, thereby compromising the cytoplasmic immunostaining. The metal intensified DAB produced a highly visible granular HRP reaction product within nerve cell bodies. The reaction product did not completely fill the perikaryon of labelled cells, and therefore allowed detection of the immunoreactivity in retrogradely labelled cells (see 47).

DISCUSSION

The problem of spurious vs. retrograde labelling.

Labelling of nerve cell bodies and processes within the auditory brainstem can occur in a number of ways after injections into the cochlea. Conventional anterograde and retrograde transport occurs, leading to staining of primary afferent terminals within the CN and of cochlear efferent cell bodies in the SOC. Neurons can also become labelled by trans-neuronal transport, but this requires relatively long survival times and is not observed within 48 hours after cochlear injections of free HRP. The tracer may, however, contaminate the CSF via the periotic duct or, as Ross *et al.* (38) has proposed, by spread over the eighth nerve sheaths. This contamination can lead to spurious cellular labelling due to direct access to the interstitial fluid of the brainstem, either via a perivascular avenue (34) or simply by general diffusion. This spurious labelling is difficult to prevent. Although Bergmann *et al.* (7) and Warr (48) sealed the periotic duct in guinea pigs and kittens, we were not able to accomplish this in rats (unpublished experiments). Our results, however, showed that this procedure is not necessary because CSF contamination seems to be a problem only after cochlear injections of WGA-HRP or large amounts of free HRP.

Despite the possibility that free HRP leaks from the cochlea into the CSF, there are compelling reasons to believe that within 48 hours after intracochlear injections of moderate amounts of this tracer, neurons in the brainstem containing HRP are so labelled due to retrograde transport and that the labelled auditory brainstem neurons are OC neurons. When few neurons are spuriously labelled,

they occur only in nuclei situated near the brainstem surface or are neurons which send processes that reach very close to the surface. If there is massive CSF contamination, most brainstem nuclei contain labelled neurons. However, neither case is observed after moderate cochlear injections of free HRP. CSF contamination with free HRP does not necessarily lead to brainstem surface staining, but always leads to NTB staining. After intracochlear HRP injections, neither the surface of the brainstem nor cells within NTB become labelled and the MVPO and LVPO are the only nuclei close to the brainstem surface which contain labelled cells. Labelling of neurons in LVPO and MVPO after moderate cochlear injections of free HRP is therefore genuine. Finally, different tracers used by other investigators without any reported problems label neurons in the same nuclei we report as containing OC neurons. Such tracers, however, were not appropriate for our double labelling procedure.

We consider the NTB staining that occurs in rats after cochlear injections of WGA-HRP to be an artifact for various reasons. NTB neurons provide numerous axon terminals to the superior olive region (J. C. Adams, personal communication) and these may constitute a conspicuous surface for tracer uptake. Moreover, Golgi methods reveal that the most ventrally located NTB neurons possess some dendrites that are oriented perpendicular to the fibers of the trapezoid body and reach near the surface of the brainstem (26, in cats, and our unpublished observations in rats). These features may explain why the ventral NTB neurons are the most prone to become spuriously labelled by contaminated CSF and interstitial fluid. Although a subset of the efferent cell population in cats is located within the territory of NTB, as are some AChE positive neurons, there has not been a previous report of NTB labelling in rats after cochlear injections and there are no AChE (32) or ChAT positive cells in the rat NTB. One must conclude that the NTB in rats does not contain OC neurons, at least that can be revealed by the present methods, and therefore any neuronal labelling in NTB after cochlear injections of HRP is a sign of spurious labelling.

There are various mechanisms which make WGA-HRP prone to spuriously label neurons after even moderate amounts are injected into the cochlea. The adhesive properties of WGA, which adheres to cell surface N-acetyl-D-glucosamine and sialic acid residues (23, 28, 39) imparts resistivity to clearance from the brainstem surface. Wheat germ agglutinin also increases the speed with which its conjugated HRP is interiorized (40), thereby decreasing the time during which CSF clearance can occur before cellular uptake begins. Paradoxically, while WGA-HRP is a more sensitive tract tracer than free HRP (18), it can give less clear results.

Free HRP suffers from none of the above problems and we have demonstrated that small amounts of HRP can even be placed in the CSF without creating spurious labelling. However, when a large amount of free HRP is present in the sub-arachnoid space, spurious labelling can occur, as demonstrated after the large cotton pellet soaked with HRP and bolus injections of HRP were placed into the CM. This presumably occurs because not enough tracer can be removed from the brainstem surface by normal CSF flow before the interstitial fluid be-

comes contaminated as well, leading to cellular uptake. The cochlea cannot be excessively loaded with free HRP (nor perhaps with other tracers: see 9) without risking spurious labelling in the brainstem, as demonstrated in the results obtained by Ross *et al.* (38) after perfusing 900 μl of 1% HRP into the cochlea. Consequently, without blocking all exit paths, which would be quite difficult, one cannot attempt to maximize retrograde labelling by injecting larger amounts of tracer into the cochlea. Instead, better tracers must be employed.

Location of efferent neurons.

We have observed HRP labelled cochlear efferent neurons in all areas previously described as containing these cells in rats (51). Large cell bodies were found bilaterally in the MVPO (except for the most caudal portions) and the RPO including its rostral-most portions where it becomes contiguous with the ventral nucleus of the lateral lemniscus (VLL). These bilateral elements represent the large cell medial efferent system described by White and Warr (51). Also, small cells of the lateral efferent system were found in the body of the ipsilateral LSO (see also 47). We add to the above list three new findings. We have noted the presence of some large efferent neurons around the margin of the medial limb of the ipsilateral LSO that have not been previously described in rat, although Helfert *et al.* (19) did describe large OC cells near the medial limb of the gerbil LSO. Also, LVPO neurons are labelled, albeit only in small numbers. Finally, we have observed some labelled small neurons in MVPO and RPO, but such cells are very few in number. These groups of neurons are not labelled after small amounts of free HRP are delivered to the CM. In addition, Osen *et al.* (32) reported the presence of large AChE-positive neurons (an indicator used by many previous investigators to establish whether an area in SOC contains cochlear efferent cells) in the LVPO and on the fiber capsule border of the LSO in rats. We therefore conclude that when free HRP is injected into the cochlea, cellular labelling in LVPO and dorsal to the medial limb of the ipsilateral LSO originates from HRP uptake by OC terminals in the organ of Corti (but see 51). These regions should therefore be included as SOC areas containing cochlear efferent neurons in rats.

The retrogradely labelled cells surrounding the ipsilateral LSO and in LVPO may belong to the medial olivocochlear system because: 1) they are large cells; and 2) they are located outside LSO (see also 47). It is difficult to be absolutely certain of such a classification, however, because the cells in question are located lateral to MSO, in a region previously defined as containing neurons only of the lateral olivocochlear system (48, 49) and because of their unknown projections within the cochlea. In any case, there are very few cells around LSO and in LVPO that project to the cochlea in rats. Perhaps it is more instructive to classify OCB neurons into large cell groups (located in MVPO, RPO, and surrounding LSO, including those in LVPO) and a small cell group (primarily located within LSO, with a rare small cell in RPO and MVPO: see 47).

Our results suggest that most OC neurons in the rat LSO are located in the medial limb. The number of labelled cochlear efferent cells progressively decreases in the middle and lateral limbs. This is in agreement with results reported by Robertson *et al.* (37) in guinea pigs and the observations of Helfert *et al.* (19) in gerbils. An alternative explanation for the medial to lateral gradient in the labelling of LSO cochlear efferent neurons could be that our HRP injections in some way produce a bias in that the fibers of the basal cochlear coil are more completely labelled than the fibers of the more apical coils. We consider this possibility less likely because the labelling pattern was consistent across experiments and also because complete cochlear filling was evident during each HRP injection.

If the tonotopic arrangement of rat efferent neurons is similar to that of guinea pigs, one could expect that the base of the cochlea contains more efferent terminals than the apex. No data are available on the numbers of efferent terminals in different turns of the cochlea. This problem is considered further in the report by Vetter *et al.* (47).

Other methodological considerations.

To produce a neurochemical map of chemically distinct cochlear efferent neurons, the retrograde tracer should label a large proportion, if not all, of the neurons in the system, many of which have small cell bodies with a thin rim of cytoplasm, and remain intact after perfusion of fixatives designed to optimize immunocytochemical staining. Also, the tracer should not quench or be quenched by the chromogen used for immunocytochemical staining.

Our data indicate that in double labelling small OC neurons, the balance between desired immunostaining and amount of detectable retrogradely transported HRP is particularly difficult to obtain. The pitfalls related to the use of WGA-HRP as a tracer for cochlear efferent neurons prevented its use for our double label experiments. Free HRP delivered in moderate amounts, however, gave satisfactory results. Conclusive results were also obtained with the combination of zinc salicylate formol fixative for optimizing immunocytochemical staining and heavy metal intensified DAB for HRP visualization. In a related paper (47), we demonstrate that this protocol is sufficient to map two sub-populations of OC neurons which may use GABA and ACh as neurotransmitters, respectively. Some problems in dealing with individual neurons, especially those with small cell bodies, may require further methodological advances.

SUMMARY

We have compared retrograde labelling of rat olivocochlear neurons after unilateral cochlear injections of wheatgerm agglutinin conjugated horseradish peroxidase (WGA-HRP) and free HRP. After cochlear injection of WGA-HRP, labelling of nerve

cell bodies in the brainstem can be explained not only as conventional retrograde labelling resulting from uptake by efferent nerve terminals synapsing on or near hair cells, but also as spurious labelling originating from tracer leakage, through the periotic duct and over the eighth nerve sheaths, into the cerebral-spinal fluid. Depending on the length of survival time, spurious labelling can involve small portions of the nucleus of the trapezoid body or the entire auditory brainstem and other non-auditory centers.

On the contrary, moderate amounts of free HRP delivered to the cochlea do not lead to spurious labelling. With free HRP as the tracer of choice, we found that cochlear efferent cells were located not only in the ipsilateral LSO body and bilaterally within MVPO and RPO as already described by White and Warr (51), but also surrounding the ipsilateral LSO and in the ipsilateral LVPO. The allocation of these newly described olivocochlear neurons to the medial large cell or lateral small cell system is uncertain because they are located laterally in the brainstem and project ipsilaterally but are large spherical to fusiform or multipolar cells.

A zinc salicylate-formol fixative and a metal intensified DAB reaction were found to be effective in visualizing retrogradely transported HRP in neurons and allowed immunocytochemical staining of the same sections with antisera to glutamic acid decarboxylase and choline acetyltransferase. This double label protocol can be used to produce a neurochemical map of the OC systems.

Acknowledgements. — This work was supported by US-PHS grant No. 09904 (E.M.). We thank Drs. K. K. Osen, J. C. Adams, and A. Berrebi for their thoughtful comments and suggestions.

REFERENCES

1. Abercrombie, M. Estimation of nuclear population from microtome sections. *Anat. Rec.*, **94**: 239-247, 1946.
2. Adams, J. C. Heavy metal intensification of DAB-based HRP reaction product. *J. Histochem. Cytochem.*, **29**: 775, 1981.
3. Adams, J. C. Cytology of periolivary cells and the organization of their projections in the cat. *J. Comp. Neurol.*, **215**: 275-289, 1983.
4. Aschoff, A. and Ostwald, J. Different origins of cochlear efferents in some bat species, rats, and guinea pigs. *J. Comp. Neurol.*, **264**: 56-72, 1987.
5. Aschoff, A. and Ostwald, J. Distribution of cochlear efferents and olivo-collicular neurons in the brainstem of rat and guinea pig. *Exp. Brain Res.*, **71**: 241-251, 1988.
6. Aschoff, A., Muller, M. and Ott, H. Origins of cochlear efferents in some gerbil species. *Exp. Brain Res.*, **71**: 252-261, 1988.
7. Bergmann, K., Haupt, H., Scheibe, F. and Rogge, I. Der Verschluß des Aquaductus Cochleae für Perilymphuntersuchungen am Meerschweinchen. *Arch. Otorhinolaryngol.*, **224**: 257-265, 1979.
8. Bishop, A. L. and Henson, O. W. Jr. The efferent cochlear projections of the superior olivary complex in the mustached bat. *Hearing Res.*, **31**: 175-182, 1987.
9. Borges, C. F., Elliot, P. J., Iversen, S. D. and Iversen, L. L. Selective extraction

of large and small molecules from the cerebrospinal fluid by Purkinje cells. *Science*, **228**: 346-348, 1985.

10. BOWKER, R. M., WESTLUND, K. N., SULLIVAN, M. C. and COULTER, J. D. A combined retrograde transport and immunocytochemical staining method for demonstrating the origins of serotonergic projections. *J. Histochem. Cytochem.*, **30**: 805-810, 1982.
11. CAMPBELL, J. P. and HENSON, M. M. Olivocochlear neurons in the brainstem of the mouse. *Hearing Res.*, **35**: 271-274, 1988.
12. CONTRERAS, R. J., GOMEZ, M. M. and NORGREN, R. Central origins of cranial nerve parasympathetic neurons in the rat. *J. Comp. Neurol.*, **190**: 373-394, 1980.
13. DIERINGER, N., BLANKS, R. H. I. and PRECHT, W. Cat efferent vestibular system: Weak suppression of primary afferent activity. *Neurosci. Lett.*, **5**: 285-290, 1977.
14. DUCKERT, L. The morphology of the cochlear aqueduct and periotic duct of the guinea pig- a light and electron microscopic study. *Trans. Amer. Acad. Ophth. Otol.*, **78**: ORL 21-ORL 48, 1974.
15. DUVALL, A. J. and SUTHERLAND, C. R. Cochlear transport of horseradish peroxidase. *Ann. Otol.*, **81**: 705-713, 1972.
16. FEX, J. and ALTSCHULER, R. A. Neurotransmitter-related immunocytochemistry of the organ of Corti. *Hearing Res.*, **22**: 249-263, 1986.
17. GOLDBERG, J. M. and FERNANDEZ, C. Efferent vestibular system in the squirrel monkey: Anatomical location and influence on afferent activity. *J. Neurophysiol.*, **43**: 986-1025, 1980.
18. GONATAS, N. K., HARPER, C., MIZUTANI, T. and GONATAS, J. O. Superior sensivity of conjugates of horseradish peroxidase with wheat germ agglutinin for studies of retrograde axonal transport. *J. Histochem. Cytochem.*, **27**: 728-734, 1979.
19. HELFERT, R. H., SCHWARTZ, I. R. and RYAN, A. F. Ultrastructural characterization of gerbil olivocochlear neurons based on differential uptake of ^{3}H-D-aspartic acid and wheatgerm agglutinin- horseradish peroxidase conjugate from the cochlea. *J. Neurosci.*, **8**: 3111-3128, 1988.
20. HÖKFELT, T., SKAGERBERG, G., SKIRBOLL, L. and BJÖRKLUND, A. Combination of retrograde tracing and neurotransmitter histochemistry. Pp. 228-285. In: BJÖRKLUND A. and HÖKFELT, T. (Eds.), *Handbook of Neuroanatomy. Vol. 1: Methods in Chemical Neuroanatomy*. New York, Elsevier Science, 1983.
21. HORN, A. K. E. and HOFFMANN, K.-P. Combined GABA-immunocytochemistry and TMB-HRP histochemistry of pretectal nuclei projecting to the inferior olive in rats, cats and monkeys. *Brain Res.*, **409**: 133-138, 1987.
22. LJUNGDAHL, A., HÖKFELT, T., GOLDSTEIN, M. and PARK, D. Retrograde peroxidase tracing of neurons combined with transmitter histochemistry. *Brain Res.*, **84**: 313-319, 1975.
23. MAGET-DANA, R., ROCHE, A. C. and MONSIGNY, M. Interaction between vesicles containing gangliosides and lectins. *FEBS Lett.*, **79**: 305-309, 1977.
24. MESULAM, M.-M. and ROSENE, D. L. Sensitivity in horseradish peroxidase neurohistochemistry: a comparative and quantitative analysis of nine methods. *J. Histochem. Cytochem.*, **27**: 763-773, 1979.
25. MESULAM, M.-M. Principles of horseradish peroxidase neurohistochemistry and their applications for tracing neural pathways- axonal transport, enzyme histochemistry and light microscopic analysis. Pp. 1-151. In: MESULAM, M.-M. (Ed.), *Tracing Neural Connections with Horseradish Peroxidase*. New York, John Wiley and Sons, 1982.
26. MOREST, D. K. The collateral system of the medial nucleus of the trapezoid body of the cat, its neuronal architecture and relation to the olivo-cochlear bundle. *Brain Res.*, **9**: 288-311, 1968.
27. MUGNAINI, E. and DAHL, A.-L. Zinc-aldehyde fixation for light microscopic immunocytochemistry of nervous tissues. *J. Histochem. Cytochem.*, **31**: 1435-1438, 1983.

28. NICOLSON, G. L. The interactions of lectins with animal cell surfaces. *Int. Rev. Cytol.*, **39**: 89-190, 1974.
29. OERTEL, W. H., SCHMECHEL, D. E., TAPPAZ, M. L. and KOPIN, I. J. Production of a specific antiserum to rat brain glutamic acid decarboxylase by injection of an antigen-antibody complex. *Neuroscience*, **6**: 2689-2700, 1981.
30. OERTEL, W. H., MUGNAINI, E., SCHMECHEL, D. E., TAPPAZ, M. L. and KOPIN, I. J. The immunocytochemical demonstration of GABAergic neurons- methods and application. Pp. 297-329. In: CHAN-PALAY, V. and PALAY, S. (Eds.), *Cytochemical Methods in Neuroanatomy*. New York, A. R. Liss, 1982.
31. ORDRONNEAU, P., LINDSTRÖM, P. B.-M. and PETRUSZ, P. Four unlabelled antibody bridge techniques: A comparison. *J. Histochem. Cytochem.*, **29**: 1397-1404, 1981.
32. OSEN, K. K., MUGNAINI, E., DAHL, A.-L. and CHRISTIANSEN, A. H. Histochemical localization of acetylcholinesterase in the cochlear and superior olivary nuclei. A reappraisal with emphasis on the cochlear granule cell system. *Arch. Ital. Biol.*, **122**: 169-212, 1984.
33. PERLMAN, H. B. and LINDSAY, J. R. Relation of the internal ear spaces to the meninges. *Arch. Otoralyngol.*, **29**: 12-23, 1939.
34. RENNELS, M. L., GREGORY, T. F., BLAUMANIS, O. R., FUJIMOTO, K. and GRADY, P. A. Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. *Brain Res.*, **326**: 47-63, 1985.
35. RHODES, C. H., STIEBER, A. and GONATAS, N. K. Transneuronally transported wheat germ agglutinin labels glia as well as neurons in the rat visual system. *J. Comp. Neurol.*, **261**: 460-465, 1987.
36. ROBERTSON, D. Brainstem location of efferent neurones projecting to the guinea pig cochlea. *Hearing Res.*, **20**: 79-84, 1985.
37. ROBERTSON, D., ANDERSON, C.-J. and COLE, K. S. Segregation of efferent projections to different turns of the guinea pig cochlea. *Hearing Res.*, **25**: 69-76, 1987.
38. ROSS, M., NUTTALL, A. L. and WRIGHT, C. G. Horseradish peroxidase acute ototoxicity and the uptake and movement of the peroxidase in the auditory system of the guinea pig. *Acta Otolaryngol.*, **84**: 187-201, 1977.
39. SCHWAB, M. E., JAVOY-AGID, F. and AGID, Y. Labelled wheat germ agglutinin (WGA) as a new, highly sensitive retrograde tracer in the rat brain hippocampal system. *Brain Res.*, **152**: 145-150, 1978.
40. SILVERSTEIN, S. C., STEINMAN, R. M. and COHN, Z. A. Endocytosis. *Ann. Rev. Biochem.*, **46**: 669-722, 1977.
41. STAINS, W. A., KIMURA, H., FIBIGER, H. C. and MCGEER, E. G. Peroxidase labelled lectin as a neuroanatomical tracer: evaluation in a CNS pathway. *Brain Res.*, **197**: 485-490, 1980.
42. STRUTZ, J. Efferent innervation of the cochlea. *Ann. Otol.*, **90**: 158-160, 1981.
43. STRUTZ, J. and BIELENBERG, K. Efferent acoustic neurons within the lateral superior olivary nucleus of the guinea pig. *Brain Res.*, **299**: 174-177, 1984.
44. STRUTZ, J. and SPATZ, W. B. Superior olivary and extraolivary origin of centrifugal innervation of the cochlea in guinea pig. A horseradish peroxidase study. *Neurosci. Lett.*, **17**: 227-230, 1980.
45. THOMPSON, G. C. and THOMPSON, A. M. Olivocochlear neurons in the squirrel monkey brainstem. *J. Comp. Neurol.*, **254**: 246-258, 1986.
46. VETTER, D. E., ADAMS, J. C. and MUGNAINI, E. A dual efferent GABAergic projection to the rat cochlea. *Soc. Neurosci. Abstr.*, **12**: 779, 1986.
47. VETTER, D. E., ADAMS, J. C. and MUGNAINI, E. Chemically distinct rat olivocochlear neurons. Submitted to *Synapse*.
48. WARR, W. B. Olivocochlear and vestibular efferent neurons of the feline brainstem:

their location, morphology and number determined by retrograde axonal transport and acetylcholinesterase histochemistry. *J. Comp. Neurol.*, **161**: 159-182, 1975.

49. WARR, W. B. and GUINAN, J. J. JR. Efferent innervation of the organ of Corti: two separate systems. *Brain Res.*, **173**: 152-155, 1979.
50. WARR, W. B., DE OLMOS, J. and HEIMER, L. Horseradish peroxidase- the basic procedure. Pp. 207-262. In: HEIMER, L. and ROBARDS. M. (Eds.), *Neuroanatomical Tract-Tracing Methods*. New York, Plenum Press, 1982.
51. WHITE, J. S. and WARR, W. B. The dual origins of the olivocochlear bundle in the albino rat. *J. Comp. Neurol.*, **219**: 203-214, 1983.
52. WILLIAMS, R. W. and RAKIC, P. Three-dimensional counting: An accurate and direct method to estimate numbers of cells in sectioned material. *J. Comp. Neurol.*, **278**: 344-352, 1988.