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The Mammalian Olivocochlear System—A Legacy of Non-cerebellar Research in the Mugnaini Lab

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Abstract

Although the major emphasis of Enrico Mugnaini's research has been on investigations of the cerebellum, a significant amount of work over a relatively short span of time was also done in his lab on a number of other brain systems. These centered on sensory systems. One of these extra-cerebellar systems that he embraced was the auditory system. Portions of the cochlear nucleus, the first synaptic relay station along the central auditory pathways, possess a cerebellar-like circuitry and neurochemistry, and this no doubt lured Enrico into the auditory field. As new tools became available to pursue neuroanatomical research in general, which included a novel antibody to glutamic acid decarboxylase (GAD), Enrico's lab soon branched out into investigating many other brain structures beyond the cerebellum, with an overall goal of producing a map illustrating GAD expression in the brain. In collaboration with long-term colleagues, one of these many non-cerebellar regions he took an interest in was an efferent pathway originating in the superior olive and projecting to the cochlea, the peripheral end organ for hearing. There was a need for a more complete neurochemical map of this olivocochlear efferent system, and armed with new antibodies and well-established tract tracing tools, together we set out to further explore this system. This short review describes the work done with Enrico on the olivocochlear system of rodents, and also continues the story beyond Enrico's lab to reveal how the work done in his lab fits into the larger scheme of current, ongoing research into the olivocochlear system.

Keywords

Enrico Mugnaini; Olivocochlear system; Cochlear efferent system; Cochlea; Hair cell innervation

Introduction

On the occasion of Enrico Mugnaini's retirement, an attempt is made here to describe work done in his lab on the olivocochlear (OC) system, a feedback projection from the brainstem auditory nuclei to the spiral ganglion cells and hair cells of the cochlea (Fig. 1). An attempt has been made to put the work done in the lab into the context of what we knew at the time we pursued this line of research, and finally how this work segued into the "post-Mugnaini

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Conflict of Interest

The author declares no conflict of interest exists.

lab” phases of investigations into the system. This helps demonstrate the environment within which decision processes were made in Enrico’s research, and illustrates the legacy of the OC work done in his lab. This will hopefully illustrate the long-lasting impact that Enrico has had on those of us fortunate enough to call him mentor and friend. This also serves to alert the reader that while we will review some of the literature concerning the olivocochlear system, this endeavor is inherently biased and based on work that can ultimately be traced back, in some manner, to Enrico’s influence. Thus, by design, many interesting contributions made by other important players in the field are mentioned briefly, or perhaps not at all. It is not the intent here to suggest, by omission, the work of others is to be ignored or in any way trivialized, and the reader is encouraged to look at any of the papers cited for follow-up material on the subject. Finally, this is a personal tribute to Enrico, and I hope the sometimes familiar, non-scientific writing style is not too offensive.

The Mugnaini Lab of the 1980s

Even a simple recounting of time spent in the Mugnaini Lab and the projects that piqued Enrico’s interests, and thereby which flowed to those of us working in the lab, must take into account the force of Enrico’s personality, his wide-ranging interests, and the high expectations for technical and intellectual excellence conveyed to those of us carrying out the work. Expectations were explicitly taught at the double-headed microscope next to Enrico’s office. Time on that microscope took on major significance, as this was where students had to demonstrate their knowledge of the material being examined, and nothing was off limits if it was visible (or perhaps even not visible!) in the field. One never knew what questions would follow, but the message was simple—know your work and be able to convey a deep understanding of the material, the techniques used to produce the results, and where one goes next.

It is important to recognize that during the 1980s, the Mugnaini lab was characterized by numerous projects beyond those Enrico is known best for—his long-term investigations of the cerebellum and its circuitry. These extra-cerebellar projects included examinations of the olfactory bulb [38, 39, 67], the striatum and amygdala [43, 45, 46], oligodendrocytes [32–34], general studies on electric fish [31, 36, 37], and of course his examinations of the central auditory system nuclei (which is described by others in this volume). Much of the expansion in research interests beyond the cerebellum came on the heels of the development of the first anti-glutamic acid decarboxylase (GAD) antibody by Wolfgang Oertel [44], with whom Enrico enjoyed a fruitful collaboration. This new tool allowed novel neuroanatomical investigations into inhibitory circuits (neurons and their projections) throughout the brain.

The Olivocochlear System—Background Leading to Investigations Undertaken in the Mugnaini Lab

Discovery and Early Studies on Localization of OC System Neurons

Previous to our work on the OC system, numerous investigations had demonstrated that OC system neurons reside within the nuclei of the superior olivary complex. In a series of reports, first in abstract form in 1942, and then as full papers in 1946 and 1953, Rasmussen

used the Marchi technique to selectively visualize and trace anterograde degenerating myelin and axons resulting from lesions made in the superior olivary complex [47–49]. Interestingly, although this was the first experimental evidence of a descending input to the inner ear, this was not the first description of descending input potentially innervating the auditory/vestibular periphery. In fact, the idea of a descending innervation potentially targeting the cochlea was forwarded by Arnold in his dissertation of 1826, and further explored in 1851. Additionally, Held reported connections to the auditory periphery in 1897 (see [50]). Yet, for the next 30 years following Rasmussen's work, the function, if not the existence, of the system was hotly debated, with alternate viewpoints being that the system was autonomic (e.g., [50]). Indeed, the prominent players supporting a neural role for the efferent system were suggested by those in opposition to hold the definition of a tragedy as quoted by T.H. Huxley: the idea of a tragedy was a deduction (read: the neural nature of the OC system) killed by a fact! Which fact was evident that should dissuade one from the neural nature of the OC system was less clear than perhaps the proponents of the non-neural nature of the OC system themselves recognized. Nonetheless, investigations continued into the neural nature of the system.

In attempts to more precisely localize the cell bodies of origin for the OC system, Rossi and Cortesina [52], and later Brown and Howlett [4], followed the logic that since the OC fibers could be differentiated from afferent cochlear fibers by their high acetylcholinesterase (AChE) levels [51], the cell bodies should also be heavily AChE-positive. This research was extremely useful in narrowing down those nuclei of the superior olivary complex that most likely contained OC neurons, but did not unambiguously delineate the cells of origin. Revealing the precise localization of the cell bodies giving rise to the fibers and terminals of the OC system awaited the evolution of new neuroanatomical techniques.

Localization of OC Neurons

The development of horseradish peroxidase (HRP) as a tract tracer [21–28] allowed tracing of neural connections by way of retrograde axonal transport from the site of injection. This technique finally enabled researchers to map with confidence the cell bodies giving rise to the OC fibers [64]. Soon after, it became apparent that the neuroanatomy of the OC system varied between species. However, a significant step forward in understanding the OC system came with the realization that the OC system could generally be divided into two zones based on the position of the cell bodies with respect to the medial superior olive (MSO) [17, 18]. The lateral olivocochlear system (LOCS) was lateral to the MSO, and was a mostly ipsilateral system (with some relatively small contralateral contribution, depending on species) located either in (rodents) or directly around and in the hilus (cat) of the lateral superior olive (LSO). The cell bodies of origin for the LOCS were found to terminate under the inner hair cells (IHCs), directly on the SGN dendrites (Fig. 1) and, in adults, to a far lesser degree also on the IHC itself. The medial olivocochlear system (MOCS) was defined as medial to MSO, and represented Rasmussen's original crossed, bilateral system. The cell bodies of origin for the MOCS were located in the ventral nucleus of the trapezoid body (VNTB), and terminated directly on the OHCs (Fig. 1). This system seemed to be fixed in its location among mammals except for the bat species *Rhinolophus rouxi* (the horseshoe bat). *R. rouxi* was found to possess a single, ipsilateral, AChE-positive group of neurons

projecting to the cochlea that were situated between the LSO and the VNTB [3]. This nucleus was termed the nucleus olivocochlearis, and matched well with the fact that *R. rouxi* is the only mammal that does not possess OC innervation of its OHCs. Thus, the OC system was found to be a complex system with respect to the location of cells of origin and the origins of the synaptic terminations on their target. Additionally, and not surprisingly, the structure of the OC system seemed to be influenced to some degree by the auditory environment the subject was required to attend to. Anyone who knows Enrico Mugnaini knows that this is the type of system he appreciates. Understanding how the function of a system dictates its form was always the kind of effort that was close to Enrico's heart, and explains his fascination with cross-species comparisons.

Neurochemistry of the OC System

As described above, the OC system of mammals is composed of neurons situated in various superior olivary nuclei. Once the cell bodies of the OC system were mapped, more attention turned to the neurochemistry of the system. Prior to our investigations, the OC system was known to be largely cholinergic. To date, AChE histochemistry had been used to reveal the OC system and map its cholinergic arm. Yet, evidence existed that within the OC system there was some small element of a GABAergic transmission system as was first delineated biochemically [14]. This early report suggested that GABA was not likely to be a neurotransmitter of the OC system based on the low GAD activity measured (approximately 1/10th that of choline acetyltransferase, ChAT). This was a curious finding given that efferent activity of the OC system inhibited cochlear function and elevated auditory thresholds. Perhaps more confounding, evidence existed that the cholinergic neurotransmission itself may underlie the inhibition [11, 12]. Ultimately, the use of the Oertel anti-GAD antibody (1440) revealed the presence of GAD-positive synaptic terminals in the cochlea of guinea pig and rat [2, 13, 15], enhancing the hypothesis that more than acetylcholine exists as an OC neurotransmitter. Functionality of the GABAergic system in the cochlea remains a topic of debate to this day, although data demonstrate some developmental/maintenance-like roles as well as a role in contributing to normal auditory thresholds [30].

The Beginnings of a Neurochemical Map of the OC System

Based on the findings that GAD immunoreactive terminals were observed under both the IHCs and the OHCs, one could easily envision GAD-positive OC neurons in both the LOCS and MOCS, but no such direct evidence had been offered. This gap in the knowledge concerning the neurochemical properties of the OC system was recognized exactly at the time the initial anti-GAD antibody immunohistochemistry protocols were worked out in the Mugnaini lab. Coincidentally, I was a new graduate student in the lab using the GAD immunostaining to explore inhibitory cells and connections of the inferior colliculus, which we soon found out was also being pursued by Enrico's long-time collaborator Kirsten Osen and her colleague Hilde Faye-Lund. This gave Enrico an opening to switch to what appeared to be fertile territory, and the olivocochlear projects ensued.

Research into the OC System During the Mugnaini Years

(i) Neurochemical mapping of the olivocochlear system

We originally set out to produce a high quality neurochemical map of the OC system in rats. This entailed combining retrograde tracing with HRP injections into the cochlea, coupled with standard immunohistochemical procedures carried out on frozen sections of the brainstem regions containing the superior olivary complex. While wheat-germ agglutinin-conjugated HRP (WGA-HRP) had previously been shown to be more sensitive than free HRP in revealing greater numbers of retrogradely labeled neurons, our initial experience with WGA-HRP injections into the cochlea revealed a significant amount of artifactual labeling of neurons near the ventral surface of the brainstem (Fig. 2) [56]. This spurious label was likely due to spread of large injections of WGA-HRP through the cochlear aqueduct and emptying of tracer into the CSF, from which the tracer was taken up directly by neurons in close proximity to the surface. Additionally, more deeply disposed neurons were also labeled, and control injections into the cisterna magna revealed that these, too, were labeled due to uptake from the CSF, likely penetrating in along blood vessels. Because all of the nuclei already known to harbor OC neurons are close to the ventral surface of the brainstem, WGA-HRP was not the best tool for the job. Settling on the use of free HRP, injections were made into the cochlea and time allowed to pass for retrograde transport. After 2 to 3 days, the brains were processed for HRP, followed by GAD and ChAT immunostaining on alternate sections. This work produced the first neurochemical mapping of OC neurons [58], and, as often happens in science, also produced surprises. First, the data clearly indicated that the MOCS was exclusively cholinergic, while the LOCS was composed of two distinctly different cell populations: ChAT-positive (presumably cholinergic) neurons and GAD-positive (presumably GABAergic) neurons. Working with Dr. Joe Adams, we also showed that the ChAT-positive cell bodies of origin of the LOCS expressed calcitonin gene-related peptide (CGRP), a potential neurotransmitter/modulator previously linked with cholinergic neurotransmission [42] and shown to be expressed and functional in hair cell systems [1]. Just as in the case of GAD, CGRP was exclusively part of the LOCS, and never part of the MOCS neuronal pool. It had previously been shown that in the cochlea, GAD-positive terminals were situated below both the IHC region as well as the OHCs. Our own immunostaining data on the rat cochlea agreed with these prior studies in that we localized GAD under both IHCs and OHCs. However, our data went on to demonstrate that CGRP-positive terminals in the cochlea were also present under both the IHCs and OHCs. Thus, GAD-positive and CGRP-positive OC neurons were located only in the LOCS cell bodies of the OC system, yet sent terminals to the outer hair cells (OHC) region. This suggested that the LOCS/MOCS dichotomy perhaps did not hold for the terminal fields of the OC cells, and was only useful for neuronal positioning within the CNS. Much later, we would find that another neuropeptide, urocortin (Ucn, a member of the corticotropin-releasing hormone family of peptides) is expressed by the LOCS cell bodies, and is expressed in fibers and terminals only in the IHC region [62], upholding the original ideas of terminal fields of LOCS and MOCS. In addition to the OC neurons labeled in the expected domains of the superior olive, we found various HRP-labeled neurons scattered around the dorsal and ventral aspects of the LSO. While these had been described previously, the numbers of neurons we had managed to label from the cochlea were more

significant than one might expect given the previously published descriptions. These extra-LSO OC neurons were consistently immunolabeled only by anti-ChAT antibodies.

(ii) Advances in tract tracing tools reveals a new population of OC neurons

Following a presentation of early aspects of this work at the Massachusetts Eye and Ear Infirmary (MEEI), I had the fortuitous chance to meet and speak at length with Dr. Jim Kobler, who introduced me to labeling the OC system with cholera toxin B subunit conjugated to HRP (CTB-HRP). Dr. Kobler's work on middle ear muscles included some injection series that labeled the OC system. His sections showing the OC system retrogradely labeled with CTB-HRP were truly stunning in their degree of dendritic fill and number of neurons labeled. His genial manner and willingness to share homemade reagents, and Enrico's willingness first to send a very inexperienced student away to give a talk, and secondly to take a chance sight unseen concerning the technique and data it generated, was key to the next step in studying the rat OC system.

Using CTB-HRP first obtained from the Kobler lab, and then produced independently in the Mugnaini lab, we were able to reveal the OC system in Golgi-like detail (Fig. 3). While the location of the main components of the OC system were replicated, this technique revealed the true nature of the OC neurons previously observed around the LSO in the rat material. While using HRP-based techniques, we had counted these OC neurons as numbering approximately 20, but the CTB-HRP tracing experiments revealed these neurons to be part of an extensive network of more than 100 neurons. Sectioning the material in all three planes, as was required to meet Enrico's expectations in all things neuroanatomical, further revealed these neurons as forming a shell around the LSO. Thus came the nomenclature of shell OC neurons [57]. Years later, Dr. W. Bruce Warr showed that the shell OC neurons had entirely different innervation trajectories in the cochlea compared to the classic LOCS neurons which they surround. Their innervation pattern was also different from the MOCS axons, lending further evidence that this group of neurons is a distinct, albeit somewhat distributed, OC nucleus [65, 66].

(iii) Combining anterograde and retrograde tract tracing techniques

The next phase of investigations into the rodent OC system in the Mugnaini lab came, as perhaps expected, in the form of a close and fruitful collaboration. This was initiated first between lab members, and then presented to Enrico, who fully supported our efforts. Dr. Enrique Saldaña was working in the lab at the time as a visiting scientist from the University of Salamanca. Dr. Saldaña was very proficient at anterograde tract tracing with a relatively new compound, Phaseolus vulgaris leucoagglutinin (PHA-L). At this time, knowledge of the neural inputs to OC neurons, which would represent circuits positioned to exert control over the otherwise reflex-like OC system, was scant at best, and that of descending inputs was only guessed at. Based on knowledge that the inferior colliculus was known to send axons down to the VNTB region, within which reside the MOCS neurons, it seemed that there was a good chance we could visualize and map the neural elements that would represent descending control over the MOC feedback system. However, at least two issues surrounded the simple assignment of inferior colliculus inputs directly targeting the MOCS cell processes or somata. First, the MOCS neurons reside well above the recognized terminal

arbor field of inferior colliculus axons in the VNTB. Second, VNTB also contains numerous GAD-positive neurons, none of which are OC neurons. Thus, in the collegial atmosphere of the Mugnaini lab, we set out to investigate whether the OC system, with special attention paid to the MOCS, receives direct descending input from the inferior colliculus [59]. Because it had also been shown that the descending input to the VNTB is tonotopic [6, 53], investigating whether descending input to MOCS from the inferior colliculus also held out an enticing functional correlate that the MOCS could be arranged centrally in a precise tonotopic manner, curious for a system that spreads its axonal arbor so widely at its target. Injections of PHA-L into the central nucleus of the inferior colliculus (ICC) and CTB-HRP into the cochlea, followed by sequential dual color chromogenic reactions, revealed the expected ICC input to the MOCS dendrites. These tracing studies revealed that MOCS dendrites are contacted by *en passant* style fiber system that often was observed to climb along the length of the innervated dendrite, many times back to the OC soma, giving off numerous synaptic terminals along the way (Fig. 4). Thus, while there are likely to be many more inputs to the MOCS neurons, the descending input to the MOCS may play a more significant role than simply what their terminal numbers suggest. Finally, preliminary electron microscopic data obtained by Dr. Saldaña in collaboration with Enrico demonstrated that the descending ICC fibers making contact with dendritic profiles all possessed unequivocal features of excitatory synapses. This therefore suggested that descending input to the MOCS is used to activate the system, thereby suppressing basilar membrane mechanics via modulation of OHC motility, which itself has been described as the cellular embodiment of the cochlear amplifier [7, 29]. Higher-level control over basilar membrane mechanics carries significance in considerations of protection against noise-induced hearing loss.

(iv) Cholinergic neurons of the cochlear nucleus

A small study was also pursued in which ChAT immunoreactivity was mapped throughout the cochlear nucleus [60]. This study, while at first seemingly unaffiliated with our work on the OC system, grew out of that research. During the production of the OC neurochemical maps, immunostaining with the anti-ChAT antibodies revealed an unexpectedly large number of immunopositive cells, along with the more well-known fibers and synaptic terminals in the cochlear nucleus. A number of these fibers and terminals were presumed to be the previously described OC system collaterals that are given off to the cochlear nucleus by the MOCS fibers as they pass on their way out toward the cochlea. The ability to visualize these fibers immunohistochemically was important for a future collaboration with Dr. Chris Brown, described below.

In summary, work on the OC system in the Mugnaini lab during the 1980s and early 1990s significantly advanced our understanding of the OC system. First, a neurochemical map of the OC system cell bodies of origin that covered three different neurotransmitters (ACh, GABA, and CGRP) was established. Second, presaging the conditions surrounding the re-discovery of the unipolar brush cell as a cell class that was previously identified but not appreciated for what it was, our work re-defined a previously recognized but unappreciated small set of OC neurons as a significant third class of OC cell, the shell OC neurons. Finally, we were able to show that the MOCS received direct descending innervation from the ICC.

Along the way, new tools were always brought to bear on the investigations at hand, teaching students to become proficient in an ever-widening repertoire of techniques. This continued to prove the most useful gift bestowed to me during my time in the Mugnaini lab.

Further Efforts into the Investigation of the OC System Beyond the Mugnaini Lab—Enrico's Continued (Continuing) Influence

Techniques and the general mind set of the need for a careful, purposeful pursuit of research learned under Enrico's tutelage continued to play an important role in further efforts to understand the OC system after leaving the lab. The next advances in our understanding of the OC system followed rapidly upon the cloning, by Dr. Ana Belen Elgoyhen, and our collaborative efforts in localization of the unusual nicotinic receptor subunits, alpha9 [9] and alpha10 [10] to both the IHCs and OHCs. This work took place while we were both postdoctoral fellows in Steve Heinemann's lab at The Salk Institute for Biological Studies. While more massive than the Mugnaini lab, Steve's lab continued to carry on the collaborative (and sometimes competitive, but always anarchic!) atmosphere that worked so well in my graduate student days in the Mugnaini lab. Work from the Mugnaini lab and others had definitively shown the OC system to be largely cholinergic, but the system was also known to be inhibitory [11]. The speed of response ruled out muscarinic ACh receptors, but at the time, all other known nicotinic subunits generated only excitatory responses. A hint that the synaptic response of hair cells to ACh was more complicated than originally thought was evident in reports described by Dr. Paul Fuchs. A massive hyperpolarization of hair cells in response to ACh that was preceded by an initial depolarization of the hair cell had recently been demonstrated [16]. Thus, a typical excitatory nicotinic receptor could be involved in OC function if it was also coupled to another channel, such as a potassium channel, that ultimately hyperpolarizes the cell. Work by Dr. Belen Elgoyhen on cloning the alpha9, and in collaboration with Dr. Jim Boulter (also at the time at The Salk Institute in the Heinemann lab), the alpha10 genes, and the elucidation of their calcium permeability suggested that if a potassium channel was functionally linked to the alpha9/10 complex, it should be calcium sensitive. Dr. Didier Dulon, who at the time was visiting Dr. Alan Ryan's lab at UCSD (coincidentally across the street from The Salk), finally put the last piece of the puzzle together by demonstrating the expression of the apamin-sensitive, small conductance potassium channel, called the SK2 channel, in hair cells [8]. Thus, the cholinergic receptor subserving neurotransmission events between the descending OC axons and the hair cells was shown to be composed of the highly calcium permeable alpha9/10 nAChRs functionally linked to a calcium-sensitive potassium channel. Together, the two-channel system represents a fast inhibitory mechanism that modulates OHC function, and indirectly controls auditory sensitivity via its effects on basilar membrane mechanics (among other potential mechanisms as well).

While work proceeded on further defining the alpha9/10 physiology at the level of the receptor, a large collaboration was formed to produce and then characterize a mouse that lacks the alpha9 gene in order to probe the role of the receptor in auditory processing. It is impossible here to fully recount all of the people involved in the project, or the enormous effort and time that went into producing and characterizing the alpha9 null mouse. But the

efforts of all paid off when the data proved to be both satisfyingly expected and also surprisingly exciting [61]. Collaborating with Dr. Charlie Liberman, whom I first met during my trip to MEEI to present our early findings on the OC system mapping project, we found that the alpha9 null line presented with a functional phenotype that mirrored the physiology typically observed following lesions that resulted in complete de-efferentation of the cochlea, yet in the case of the alpha9 mutant mouse, the efferent fibers remained completely intact. Normally, wild-type mice present with diminished amplitudes of the 2f1–f2 distortion product oto-acoustic emissions (DP) amplitudes (generated by OHC activity when two pure tones are presented to the ear and representing distortion along the basilar membrane as the tones interact with each other) when the MOCS fibers are electrically activated during recording of the DPs. Activating the MOCS alters basilar membrane motion, feeding forward to decrease afferent signaling by the IHCs, which can be visualized by examining the auditory thresholds during the shock epoch. In the alpha9 null mice, electrical shocks delivered to the MOCS did not decrease the DP amplitudes, demonstrating the functional de-efferentation while the OHCs were still innervated by efferent (MOCS) fibers. Upon further examination of the alpha9 null mice, we also showed that afferent fiber thresholds were unaffected by MOCS activation. The alpha9 subunit is capable of forming homomeric receptors, but complete replication of an OHC-like nicotinic ACh activity profile required co-expression with the alpha10 subunit [10]. Later on, we showed a similar functional phenotype to that observed in the alpha9 null mice following the generation of the alpha10 null mouse line [63]. These were expected results. But it should also be recognized that the analyses of function in the absence of alpha9 or alpha10 subunits suggest that the GABAergic OC system is not intimately involved in the classic OC function.

While the functional phenotype of the alpha9, and later the alpha10, null mice was perhaps not too surprising, save for the complete lack of apparent GABAergic function, at least in classic OC physiology, a surprise came from examining the morphology of the efferent synapses in the cochlea. Ablation of either the alpha9 or alpha10 gene resulted in hypertrophied efferent synaptic terminals under the OHCs. Additionally, while in the wild-type mouse, OHCs typically are contacted by 3–5 synaptic terminals; the alpha9 and alpha10 null mice presented with generally only one or two hypertrophied synaptic terminals under OHCs (Fig. 5). This suggested that activity at the nicotinic receptor was important for establishing synaptic contacts with hair cells. These studies followed directly from the kinds of analyses previously performed in the Mugnaini lab. Later, we established that the OC activity through the alpha9 containing receptor complex played a significant role in modulating cell adhesion molecules [40], among others, and without the normal expression of adhesion molecules, synapses could be envisioned as free to explore beyond their normal limits (Fig. 6) and expand in size, effectively giving the first fibers to arrive at OHCs a competitive edge in establishing synaptic territory.

Related to the discovery of abnormal synaptic terminals under OHCs of the alpha9 null mice was an investigation of the central collaterals of the MOCS fibers done in a collaborative effort with Dr. Chris Brown of MEEI. Using AChE histochemistry on the alpha9 null mice, we investigated the morphology of the collateral system of MOCS fibers innervating the cochlear nucleus [5] previously visualized using the anti-ChAT antibodies as described above. Briefly, the results of this study demonstrated that the number of collateral branches

to the cochlear nucleus was normal in the alpha9 null mice. Additionally, the overall structure of branch terminations was similar between alpha9 null and wild-type mice. Because alpha9 and alpha10 genes are not expressed in the brain, these results suggest that the aberrant structure of synaptic terminations under OHCs in alpha9 and alpha10 null mice are driven by the lack of target-based feedback. Presumably, the cholinergic fibers ramifying within the cochlear nucleus are interacting with other nicotinic ACh receptors, which most likely include the alpha7 subunit, based on alpha-bungarotoxin binding studies [35]. Interestingly, dysfunction at the major target zone of the MOCS does not seem to feed back to alter at least the morphological state of innervation to other targets.

Perhaps Enrico's most spectral, and yet overarching influence was felt in our follow-up studies on the alpha9 null mouse line. One take-home message from time spent with Enrico was that the way to really understand something is to look at it from many different angles. Thus, understanding the role of granule cells of the cochlear nucleus meant that one should look at numerous different species, including the mountain beaver (which he tried to talk me into examining when I first entered his lab!). While the mountain beaver project ultimately was not part of our research plan, the lesson was still learned. To this end, we sought to better understand the mechanisms by which loss of the alpha9 gene generated the neuroanatomical abnormalities observed. As described above, we had already discovered abnormal expression levels of cell adhesion molecules in the cochlea following alpha9 gene ablation, but the question persisted—what else might be altered as a result of the loss of OC activity? To answer this rather open-ended question, we employed gene microarray technology [55], and examined the postnatal maturational period during which dynamic rearrangement of OC innervation to hair cells occurs [20]. Two main findings came from this approach. First, the alpha9 null mice possessed a cochlea that up until P7 was indistinguishable from the wild-type cochlea based on a global gene expression profile. However, upon exiting the period of dynamic rearrangement, the cochleae of the alpha9 null mice were significantly different from wild-type cochleae, and appeared to cluster with the wild-type cochleae of much younger ages. Secondly, loss of the alpha9 gene was accompanied by the increased expression of genes encoding GABA receptor subunits (GABA α 1 and GABA β 2) as well as GAD, the GABA synthetic enzyme targeted by the anti-GAD antibody described above. These data and those described above concerning changes in cell adhesion molecule expression were the first indications that the OC system played a role in cochlear function beyond that which simply tied to classic OC physiology. Especially important was the suggestion that an intact and functional OC system could be critical for early maturation of the cochlea, and perhaps (based on analogies with the visual system) farther “upstream” along central auditory pathways. This has been further evaluated recently using the alpha9 null mice [19].

Collaborating with Dr. John Adelman, we obtained the SK2 null mouse line with the idea that if the SK2 channel is responsible for hyperpolarizing hair cells following the momentary depolarization induced by cholinergic OC activity, ablating the channel should effectively reverse the sign of the synapse. This was an exciting prospect; where else might one so effectively alter the basic synaptic response to neurotransmission from a defined synapse? However, much to our surprise, ablation of the SK2 gene resulted in small synaptic boutons under OHCs and ultimate death/retraction of the OC fibers from the OHC region

(Fig. 7) [41]. Since this morphological phenotype was opposite that found in the alpha9 and alpha10 null mice, we next performed a simple experiment to investigate the potential for epistatic interactions between the two genes (in this case, alpha10 and SK2). Crossing the mice to produce a double null mouse demonstrated that the SK2 phenotype took precedence over the alpha9/10 phenotype, driving the structural response of OC innervation to degeneration rather than hypertrophy. It also demonstrated that silencing the nicotinic channel could not rescue the degeneration phenotype initiated by the loss of the SK2 channel. Indeed, it seemed to exacerbate the rate of degeneration. Fully appreciating the occurrence and time course of the denervation was made possible due to the anatomical and quantitative techniques honed in the Mugnaini lab. Perhaps even more surprisingly, we found that prior to loss of the efferent fibers, the OC system was essentially nonfunctional. Thus, with electrical activation of the MOCS fibers, DP amplitudes did not change. The expected reverse of the synaptic “sign” did not materialize. This result mirrored that of the alpha9 and alpha10 null mice. Following up further on this finding, we used quantitative PCR techniques to establish that in the SK2 null mice, expression levels of both alpha9 and alpha10 genes had not changed from the wild-type expression levels. Thus, the system seemed to have some kind of physiologic break on it that disallowed excitatory OC activity. This has yet to be fully explained.

A final case to be examined involved the generation of an alpha9 knock-in (KI) mouse line (generated and characterized through a collaboration headed by Paul Fuchs and Ana Belen Elgoyhen) in which a single amino acid facing the pore of the ACh-gated channel was altered to induce a constitutively active OC-like signal in OHCs [54]. Borrowing from the lessons learned in Enrico’s lab concerning the energetic infusion visiting scientists can have on a project and in the lab in general, I was happy to host Julian Taranda as a visiting student from Dr. Elgoyhen’s lab for a number of years. During his time in my lab, the alpha9KI mice were finally generated at Johns Hopkins using his targeting construct, and we received and began characterizing the line physiologically [54] with Dr. Stéphane Maison of the Liberman lab at MEEI, as well as morphologically [40]. Briefly, the outcome of these analyses was that the point mutation generated a constitutively active receptor that then produced downstream effects that were essentially opposite those observed following ablation of the alpha9 gene. Classic OC effects were significantly heightened in the KI, which then fed forward to raise auditory brainstem response (ABR) thresholds (ABRs are most often used to test the sensitivity of hearing by demonstrating the physiological threshold over various frequencies, or also to a special stimulus termed a click). Morphologically, it was shown that instead of fewer, hypertrophied OC terminals under OHCs, the alpha9 KI possessed a significantly greater number of synaptic endings under the OHCs (Fig. 8). These results completed an overarching series of experiments that quite unexpectedly demonstrated our ability to control the morphological structure of the MOCS synapse (Fig. 9).

Conclusions and an Assessment of Enrico Mugnaini's Impact on the Forward Trajectory of Research into the OC System

Research into the olivocochlear system represented a small blip in the overall tapestry of Enrico's footprint in neuroscience. Yet, as evidenced in the preceding discussion, it is clear that Enrico had a significant impact on what might be considered (to date, at least) the last major efforts to understand the basic neuromorphological characteristics of the olivocochlear system. That is not to say that all questions are answered—indeed this is far from the case. But concentrated efforts into examining the anatomical aspects of the olivocochlear system have waned since those years of work in Enrico's lab. However, I also hope that it is clear that the many lessons learned have been brought forward into what must be considered the initiation of a period of modern day molecular investigations into the olivocochlear system. Many times, the molecular aspects of any project can become divorced from the underlying anatomy. It is easy to get caught up in the “receptor-ology” of the molecular project, and forget that this is embedded in a rich environment wherein function is modulated by form, and vice versa. And yet this need not be so. I believe we have shown that a rich field of possibilities awaits those willing to combine the molecular with the anatomical. The last time I had the opportunity to speak at length with Enrico about science, he was touring the Heinemann lab with me during my postdoc years. As we talked of some of the early results continuing to come from the projects he set in motion during my graduate years with him, he quickly realized how much bigger the olivocochlear research enterprise had become, both in terms of persons interested in the system and the fantastic powers brought to bear on the system that in his lab not many years before we could not have imagined. He smiled broadly.

Acknowledgments

This paper is dedicated to the two people who were willing to take a chance. Of course, Enrico is one of these, and this should be clearly evident from the discussions above. This is also dedicated to my postdoc mentor, Steve Heinemann, who recently passed away, far too early. It should be mentioned that during my interview with Steve when I was making my case to join his lab as a postdoctoral fellow, Steve told me that he typically did not take people from labs headed by PIs he did not personally know. However, he also made it clear that during his investigations of my background and work, he was informed on more than one occasion that Enrico had the reputation of “dotting his i's and crossing his t's”. The force with which Enrico pursued his passions in Science was probably the main reason I ultimately had the chance to pursue my postdoc with Steve, and clearly set the table for the many olivocochlear adventures we had the continued pleasure to pursue over the last 20+ years since the time in Enrico's lab.

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Fig. 1.

The mammalian olivocochlear system. As is described in the text, the olivocochlear system is typically divided into a lateral olivocochlear (*LOC*) system (*blue fiber*) that targets afferent fibers (*green fiber*) under the inner hair cell (*IHC*). Medial olivocochlear (*MOC*) system fibers (*red*) cross the tunnel of Corti and innervate the outer hair cells (*OHC*) directly. Various neurotransmitters are associated with each division of the olivocochlear system as indicated in the figure. *Silhouetted in black* are the various “support cell” populations of the cochlea

Fig. 2.

Spurious labeling and the evolution of techniques to map the olivocochlear system.

Injections of WGA-HRP into the cochlea (injection side indicated by *open arrow, right side*) resulted in leakage of the tracer into the CSF, probably via the cochlear aqueduct. Once in the CSF, neurons close to the ventral surface of the brainstem, which includes numerous olivocochlear neurons, endocytose the tracer and therefore become spuriously labeled. This is one example of the evolution of techniques required to ultimately produce a neurochemical map of the olivocochlear system. (reproduced with permission from [56])

Fig. 3. Cholera toxin B-HRP tract tracing of the olivocochlear system. Linking horseradish peroxidase to the cholera toxin B subunit permitted Golgi-like filling of dendritic arbors of the olivocochlear neurons. **a** The lateral superior olive (LSO) is prominently displayed following fill of the lateral olivocochlear cell bodies. Some neurons of the medial olivocochlear system are also found in this section, indicated by *small arrowheads*. Other olivocochlear neurons ventral to the LSO, indicated by the *large arrowheads*, are located in the lateral nucleus of the trapezoid body, while a set of olivocochlear neurons also resides close to the LSO capsule, indicated by the *arrows*. **b** These olivocochlear neurons close to the LSO were found to encapsulate the medial limb of the LSO, and for reasons related to their distribution, were termed Shell Olivocochlear Neurons. The shell olivocochlear neurons are indicated by the *black arrows*, while the intrinsic olivocochlear neurons of the LSO body are indicated by the *white arrows*. The dendritic fill afforded by the CT-B HRP is readily observable in this photomicrograph. **c** By using CT-B HRP to trace the origins of the olivocochlear system, a map could be produced that then included the shell olivocochlear neurons as a significant contributor to olivocochlear anatomy. (reproduced with permission from [57])

Fig. 4.

Double labeling the olivocochlear system with CT-B HRP and Pha-L to determine the descending input to the MOCS neurons. Injections of Pha-L into the central nucleus of the inferior colliculus and CT-B HRP into the cochlea permitted the resolution of descending inputs onto MOCS neurons. **a** The terminal field of descending inferior colliculus axons is indicated by the *marked arrow*. This terminal field lies below the plane within which most MOCS neurons reside, but the fill generated by CT-B HRP permits visualization of dendrites over exceptionally long distances (*open arrowheads*). MOCS cell bodies are indicated by *labeled arrows*. **b** Camera lucida drawings demonstrate the nature of inferior colliculus axon interactions with the MOCS cell bodies. The *insert*, while not of the same cell drawn, demonstrates the same principle of innervation by the collicular fibers as demonstrated in the camera lucida drawing. These fibers crawl along MOCS cell dendrites while giving off small boutons (*arrows*), many times back to the soma itself. (reproduced with permission from Vetter et al., [59, 60])

Fig. 5. Abnormal morphology of MOCS synaptic terminals under outer hair cells. **a** Immunostaining of cochlear surface (whole mount) preparations isolated from wild-type mice reveal the normal innervation to OHCs typically consists of 3–4 synaptic boutons. **b** In $\alpha 9$ null mice, OHCs maintain their innervation by MOCS fibers, but the fibers generally produce, on average, between 1–2 synaptic terminals per OHC. This pattern was identical across all frequency regions of the cochlea. (reproduced with permission from [61])

Fig. 6.

Development of MOCS innervation to outer hair cells. The development of innervation to outer hair cells was followed between P0 and P21. Hair cells were immunolabeled with anti-Pep19 (*red*) and efferent terminals were immunolabeled with anti-synaptophysin (*green*). Illustrated is the P13 cochlea. **a** In P13 wild-type mice, MOCS terminals (*green*) have completely innervated OHCs, which is always limited to the base. **b** However, in the $\alpha 9$ null mice, MOCS innervation has not been constrained to the base of the OHCs (*arrow*), and instead was observed to encase OHCs in a trabecular network of fibers (*arrowhead*). This ectopic innervation was short-lived, however, and collapsed back to the base by P18. (*scale bars*, 10 μ m) (reproduced with permission from [40])

Fig. 7. Loss of SK2 potassium channel induces MOCS fiber degeneration. Immunostaining using anti-synaptophysin antibodies permits visualization of olivocochlear synaptic boutons and parent fibers. **a** In wild-type mice, synaptophysin immunostaining reveals the inner spiral bundle (*ISB*), which are the lateral olivocochlear innervation below the IHC region, the tunnel crossing fibers, which are the medial olivocochlear fibers traversing out toward the OHCs, and the synaptic terminals themselves, represented here as *small dot-like profiles* in the OHC region. **b** In SK2 null mice, the MOCS fibers degenerate, leaving only the ISB. In this photomicrograph, the exposure was purposely lengthened in an attempt to reveal the last few tunnel crossing fibers left, and hence the ISB seems thicker than the wild-type, which is simple photographic artifact. **c** High magnification of MOCS terminals below OHCs of wild-type mice reveals the multiple innervation pattern to each hair cell (*arrows*). **d** These synaptic terminals degenerate rapidly following the onset of hearing. *Arrows* indicate degenerating synaptic terminals. **e** The situation on the SK2 null mice can be compared to the hypertrophy (*short arrow*) that occurs in the alpha9 null mice. Note that some OHCs maintain a relatively normal population of terminals (*long arrow*). (reproduced with permission from [41])

Fig. 8. Inducing $\alpha 9$ over-activity alters innervation to outer hair cells. A point mutation at the 9' leucine generates a constitutively active $\alpha 9$ receptor. Immunostaining wild-type cochleae with anti-synaptophysin reveal the previously described innervation pattern by MOCS onto OHCs. **a** Wild-type mice possess between 2 and 3 MOCS terminals per hair cell (one set indicated by *arrow*). **b** In the $\alpha 9$ knock-in mouse, OHCs are innervated by more synaptic terminals than is normally ever observed in the wild-type state. OHCs can be innervated by 3–5 MOCS terminals. **c** A cumulative histogram demonstrates the innervation density per OHC along the middle turn of the mouse cochlea. **d** Not only are there more terminals innervating each OHC, the volume they collectively occupy under the OHCs is more than twice that of wild types. (reproduced with permission from [40])

Fig. 9.

Investigations into the morphological correlates of MOCS activity reveals its role in directing synaptic architecture. An unexpected result of investigations into the molecular aspects of MOCS development and function, which led directly from work begun in Enrico Mugnaini's lab, has led to the conclusion that manipulation of the activity of this synapse allows us to sculpt its morphological properties. Thus, loss of activity produced by ablation of either the alpha9 or alpha10 genes produces a MOCS innervation that is composed of few terminals to each OHC, and the terminals are larger than normal. If instead the system is hyper-active, the MOCS innervation is greater than normal, and is composed of larger than normal volumes of terminals. However, ablation of the functionally linked potassium channel, the SK2 channel, results in degradation of the MOCS, which cannot be rescued by ablation of the nicotinic ACh receptor