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Organ of Corti explants direct tonotopically-graded morphology of spiral ganglion neurons in vitro

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Abstract

The spiral ganglion is a compelling model system to examine how morphological form contributes to sensory function. While the ganglion is composed mainly of a single class of type I neurons that make simple one-to-one connections with inner hair cell sensory receptors, it has an elaborate overall morphological design. Specific features, such as soma size and axon outgrowth, are graded along the spiral contour of the cochlea. To begin to understand the interplay between different regulators of neuronal morphology, we co-cultured neuron explants with peripheral target tissues removed from distinct cochlear locations. Interestingly, these ‘hair cell microisolates’ were capable of both increasing and decreasing neuronal somata size, without adversely affecting survival. Moreover, axon characteristics elaborated *de novo* by the primary afferents in culture were systematically regulated by the sensory endorgan. Apparent peripheral nervous system (PNS)-like and central nervous system (CNS)-like axonal profiles were established in our co-cultures allowing an analysis of putative PNS/CNS axon length ratios. As predicted from the in vivo organization, PNS-like axon bundles elaborated by apical co-cultures were longer than their basal counterparts and this phenotype was methodically altered when neuron explants were co-cultured with microisolates from disparate cochlear regions. Thus, location-dependent signals within the organ of Corti may set the ‘address’ of neurons within the spiral ganglion, allowing them to elaborate the appropriate tonotopically-associated morphological features in order to carry out their signaling function.

Graphical Abstract

Co-cultures of spiral ganglion neurons (SGN, red) and organ of Corti (dotted line) reveal that tonotopic elements of the in vivo structure can regenerate in vitro. Additionally, a “transition zone” (arrowheads) forms that could serve to separate the distinctive PNS (line) and CNS cell phenotypes and molecular markers (synaptophysin, green).

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Keywords

spiral ganglion; soma size; axon; outgrowth; myelin; calbindin; class III β -tubulin; HCN1; MAP2; MBP; p75^{NTR}; Sortilin; synaptophysin; CBA/CaJ mice; B6.Cg-Tg(Thy1-YFP); Alexa-Fluor 488-conjugated anti-rabbit secondary antibody; Alexa-Fluor 594-conjugated anti-mouse secondary antibody; Alexa-Fluor 488 conjugated anti-mouse secondary antibody; Alexa-Fluor 594-conjugated anti-rabbit secondary antibody; Alexa-Fluor 350-conjugated anti-rabbit secondary antibody; Alexa-Fluor 350-conjugated anti-mouse secondary antibody; Alexa-Fluor 488-donkey conjugated anti-goat secondary antibody; diamidino-2-phenylindole

Introduction

The spiral ganglion, composed of primary-auditory afferent somata, conforms to its morphologically-inspired name by closely following the contours of the peripheral end organ, thus reproducing the coiled form of the cochlea. Type I neurons compose 95% of the ganglion, each synaptically connected to a single hair cell sensory receptor, thus having the capacity to receive and transmit acoustically-generated receptor signals with accuracy (Spoendlin, 1973). This information is then conducted into the CNS where axons branch to innervate separate regions within the cochlear nucleus, ultimately forming multiple frequency maps (MacLeod and Carr, 2007; Oertel, 1997; Rubel and Fritsch, 2002; Ryugo and Parks, 2003).

The peripheral and central processes of type I neurons have highly structured morphological features associated with the major sensory modality of frequency. For example, analysis of

the axonal projections from the hair cells to a prominent central synapse, the end bulb of Held, showed that while total axon length is essentially identical for all neurons (Fekete et al., 1984) the ratio of the peripheral to central axonal length is systematically graded. Specifically, apical neurons distant from the internal auditory meatus (IAM) must extend for relatively long distances through the core of the cochlea, thus having a higher peripheral to central axon length ratio. Conversely, basal neurons close to the IAM almost immediately traverse the Schwann-glia border and extend for relatively long distances through the central nerve root before the parent axon bifurcates, having a distinctly lower peripheral to central axon length ratio. Thus, this design reveals that it is the proportion of the central and peripheral regions that systematically change with tonotopy (Fekete et al., 1984; Liberman and Oliver, 1984). The experiments carried out herein show, remarkably, that axons regenerate uniquely PNS-like and CNS-like profiles *de novo* in tissue culture. This observation allows for examination of the ratio of these two axon profiles in controlled conditions in order to determine the underlying regulatory mechanisms.

Not only are the lengths of the axons graded along the cochlear contour, but even their cell bodies display size gradations. The somata of neurons in the basal region are significantly larger than those in the apical region in gerbils, cats, and humans (Echteler and Nofsinger, 2000; Liberman and Oliver, 1984; Nadol et al., 1990; Rosbe et al., 1996; Ryugo, 1992). In contrast to axonal length described above, which is clearly associated with the structural design of the cochlea relative to its CNS synaptic targets, the rationale for tonotopic soma size differences is not as straightforward. The interposition of the bipolar soma within the transmission pathway, however, indicates that it could have a significant impact on filtering electrotonic events (Robertson, 1976) as well as altering conduction velocity (Johnston et al., 1995; Lawson and Waddell, 1991).

While the functional imperatives of the tonotopically-graded PNS/CNS axon ratio and soma area may differ, these features are both precisely associated with the frequency contour of the cochlea and, shown herein, are controlled by it. Thus, we can conclude that the cochlea maintains a morphological map that is transmitted to the innervating spiral ganglion neurons.

Materials and Methods

Tissue culture

Procedures performed on CBA/CaJ mice (RRID:IMSR_JAX:000654) and two Thy1-YFP mouse strains (B6.Cg-Tg(Thy1-YFP)16Jrs/J, (RRID:IMSR_JAX:003709) Jackson Labs and Thy1-YFP12Jrs/J., generously provided by Dr. Jianxin Bao, Northeast Ohio Medical University) were approved by The Rutgers University Institutional Review Board for the Use and Care of Animals (IRB-UCA), protocol 90-073. Postnatal day 5–9 (P5-9) animals were euthanized by decapitation and inner ear tissues were prepared for paraffin-embedded sections, whole mounts consisting of the organ of Corti and innervating spiral ganglion neurons, or isolated under sterile conditions for tissue culture. All cell cultures were maintained in growth medium: DMEM (Sigma D6171) with 10% fetal bovine serum, 4mM L-glutamine, and 0.1% penicillin-streptomycin. Neurons were maintained in culture at 37°C in a humidified incubator with 5% CO₂. In order to assess changes in tonotopic identity all

culture preparations were analytically evaluated without neurotrophin supplementation. However, either BDNF (PeproTech 450-02) and / or NT-3 (PeproTech, 450-03), each at 5ng/ml, were added to enhance neuron survival in select preparations in order to allow more robust assessments of axonal outgrowth patterns.

Four different culture preparations of postnatal mouse tissue explants were utilized. In all preparations after excising the tissues from the animal we preformed all our organ of Corti and spiral ganglion microdissections under an Olympus dissecting microscope (SZH10) using fine forceps (Fine Science Tool USA Inc., cat# 11295-10) in L-15 media (Leibovitz, cat# L5520) without enzyme supplementation. Tissues were plated on 35mm culture dishes (Fisher Scientific Co, cat# 087724A) coated with poly-L-lysine (Sigma, cat# P9155-5MG). *Neuronal cultures* are composed of spiral ganglion explants removed from either the apical or basal fifth of the ganglion and maintained for 6–7 days in vitro (div) (Adamson et al., 2002b). *Gangliotopic cultures* consisted of the entire spiral ganglion, without the peripheral target tissue, placed intact in a tissue culture dish for 4 div in order to maintain the relative positions of neurons (Liu and Davis, 2007). *Organ of Corti/Spiral Ganglion/Cochlear Nucleus* cultures consisted of the peripheral endorgan and central target tissues still attached to the spiral ganglion and retained for 2–4 div to eliminate efferent innervation. *Synapse Cultures*, which were originally described in Flores-Otero et al., 2007 and are the focus of this study, were prepared from spiral ganglion neuron explants co-cultured for 10–22 div with organ of Corti microisolates that consist of the inner and outer hair cell receptors and surrounding satellite cells. Hair cell microisolate explants, containing a single row of inner hair cells, three rows of outer hair cells and associated satellite cells, were microdissected from defined regions of the organ of Corti (approximately 1/5th of the endorgan; Fig. 1A, dotted line) and paired with an equally sized and defined explant from the spiral ganglion (Fig. 1A, arrows). By removing each region separately and allowing them to regenerate *in vitro*, we can observe the effects of mixing-and-matching hair cell microisolates (H) with neuronal explants (N) from the apex (A) middle (M) or base (B) of the cochlear contour (Fig. 1B). In these preparations, peripheral processes are regenerated back to the endorgan for some, but not all neurons (Fig. 1C, arrows, asterisks). To assess outgrowth away from the peripheral target tissues we compared the length measured from the center to the furthest edge of the neuronal explant at time of plating (Fig. 1D, double arrow) to neurite outgrowth at 17 div assessed from the center of the explant (Fig. 1E, SGN) to the abrupt change in axon trajectory, termed the ‘transition zone’ (Fig. 1E, arrows). This analysis showed that the truncated explants elaborated *de novo* outgrowth both toward and away from hair cell microisolates. While there are a number of attributes to examine in these co-cultures, it is the process outgrowth away from the hair cell microisolates (Fig. 1E, double arrow) and neuronal soma size that are the focus of this study.

Paraffin embedded sections

Cochlear sections were obtained from CBA/CaJ mice. Temporal bones were removed from P5-7 mice and placed in either 10% formalin or 100% methanol for 45 min followed by three rinses with 0.01M phosphate-buffered saline (PBS, pH7.4) for 15 min or overnight at room temperature (RT). Temporal bones were dehydrated in 50%, 75%, 80% and 95% ethanol for 1 hr each, followed by 95% ethanol for an additional hour, 100% ethanol for 30

min, and n-Butanol overnight at RT. The next day, n-Butanol was replaced with fresh n-Butanol, which was applied for an additional 2 hours. Finally, temporal bones were embedded in paraffin, sectioned at 4–6 μ m thickness, placed on poly-L-lysine coated glass slides (VWR, 48311-703), dried overnight and stored at RT until ready for use.

Immunofluorescence

Tissue cultures were fixed with 100% methanol, washed and incubated at RT for 1 hr in 5% normal goat serum (NGS) or 5% powdered milk after which primary antibodies were applied and incubated for 24 hr at 4°C. After washing with 0.01 M PBS, pH 7.4, tissue cultures were incubated for 1 hr at RT with fluorescent-conjugated secondary antibody (1:100) obtained from Invitrogen (Alexa-Fluor 488-conjugated anti-rabbit secondary antibody (Molecular Probes (Invitrogen) Cat# A11070 RRID:AB_142134), Alexa-Fluor 594-conjugated anti-mouse secondary antibody (Molecular Probes (Invitrogen) Cat# A11020 RRID:AB_141974), Alexa-Fluor 488 conjugated anti-mouse secondary antibody (Molecular Probes (Invitrogen) Cat# A11017 RRID:AB_143160), Alexa-Fluor 594-conjugated anti-rabbit secondary antibody (Molecular Probes (Invitrogen) Cat# A11072 RRID:AB_142057), Alexa-Fluor 350-conjugated anti-rabbit secondary antibody (Molecular Probes (Invitrogen) Cat# A11069 RRID:AB_1500780), Alexa-Fluor 350-conjugated anti-mouse secondary antibody (Molecular Probes (Invitrogen) Cat# A11068 RRID:AB_1500741), Alexa-Fluor 488-donkey conjugated anti-goat secondary antibody (Molecular Probes (Invitrogen) Cat# A11055 RRID:AB_142672)). Tissue preparations were then washed and mounted in DABCO.

Antibody Characterization

Antibody labeling in this study was utilized to identify either specific cell types, sub-cellular areas, or to define regional specializations of the synapse culture preparations described above. A list of the antibodies utilized in the study is given in Table 1.

Anti-calbindin Antibodies—Monoclonal anti-calbindin antibody (Swant Cat# 300 RRID:AB_10000347) and polyclonal anti-calbindin antibody (Swant Cat# CB 38 RRID:AB_10000340), both at 1:100 dilution, were used to identify hair cell receptors and spiral ganglion neurons in vitro. The specificity of the both antibodies was examined in null mutant mice and conditional null mutant mice. The calbindin protein in mutant mice remained undetected in Western blot and immunostaining in comparison to wild type mice (Airaksinen et al., 1997; Barski et al., 2002; Kook et al., 2014). The specificity of polyclonal anti-calbindin antibody was further evaluated with Western blot analysis on cochlear tissues (Liu and Davis, 2014a) showing that the antibody recognized a band at the expected molecular weight of 28kDa (Schwaller, 2009).

Anti-class III β -tubulin Antibodies—Monoclonal anti-class III β -tubulin antibody (Covance Research Products Inc. Cat# MMS-435P-100 RRID:AB_663338, 1:200 dilution) or polyclonal anti-class III β -tubulin antibody (Covance Research Products Inc Cat# PRB-435P-100 RRID:AB_10063850, 1:350 dilution) were used to distinguish spiral ganglion neurons from surrounding satellite cells. Western blot analysis in rat tissues confirmed the predicated molecular weight for β -tubulin of 50kDa using the monoclonal

antibody (Johansen et al., 2014). Western blot and immunocytochemical analysis using the polyclonal antibody confirmed the lack of β -tubulin protein in ganglionectomized animals compared to control tissues (Calinescu et al., 2011). Moreover, both monoclonal and polyclonal anti- β -tubulin antibodies have been used successfully to specifically label the cytoskeleton of spiral ganglion neurons in vitro and in vivo (Flores-Otero and Davis, 2011; Flores-Otero et al., 2007; Reid et al., 2004).

Anti-HCN1 Antibody—In order to assure that labeling of the cytoskeletal protein β -tubulin accurately assessed the full extent of the neuronal soma membrane we compared its immunolabeling to that of the hyperpolarization-activated cyclic nucleotide-gated potassium channel 1 (HCN1) α -subunits that are localized to neuronal membrane in some, but not all spiral ganglion neurons (Liu et al., 2014b). The anti-HCN1 polyclonal antibody utilized in this study (Alomone Labs Cat# APC-056 RRID:AB_2039900, 1:200 dilution) targeted the peptide (C) KPNSASNSRDDGNSVYPSK which corresponds to amino acids 6–24 of rat HCN1. Western blot analysis confirmed the amino terminus of the rat HCN1 was immunolabeled with this antibody (Ramakrishnan et al., 2009). The specificity of anti-HCN1 was confirmed previously by Western blot analysis revealing a single band at the predicted molecular weight (Stradleigh et al., 2011), along with the expected lack of labeling in HCN1^{-/-} knockout mice (Herrmann et al., 2011). No cross-reactivity has been observed when HCN1 and HCN4 polyclonal antibodies were used in Western blots of lysates from HEK cells over-expressing HCN1-HCN4 (Battefeld et al., 2012).

Anti-MAP2 Antibody—Localization of microtubule-associated protein 2 (MAP2) to the spiral ganglion somata and initial processes has been previously reported (Chen et al., 2011). The polyclonal anti-MAP2 antibody (EMD Millipore Cat# AB5622 RRID:AB_91939, 1:100 dilution) utilized to label spiral ganglion somata herein recognizes all MAP2 isoforms (MAP2a, MAP2b, MAP2c and MAP2d) with the greatest affinity to MAP2a,b. Western blot analysis reveals specific antibody labeling of a MAP2a, MAP2b 280kDa doublet and a MAP2c 70kDa doublet in adult rat brain (Millipore Product Sheet). Immunocytochemical analysis further demonstrates that the antibody specifically labels dendrites of central amygdala neurons (Justice et al., 2008).

Anti-MBP Antibody—Myelin basic protein (MBP) localization within synapse cultures was utilized to determine the distribution of putative myelin segments within synapse cultures. Anti-MBP polyclonal antibody (Millipore Cat# AB980 RRID:AB_92396, 1:100 dilution) specificity analyzed by Western blot using total protein lysates from familial dysautonomia and control brains, identified two myelin basic protein isoforms at the predicated molecular weights of 25kDa and 14kDa (Cheishvili et al., 2014).

Anti-p75^{NTR} Antibody—Polyclonal antibodies against the extracellular (Millipore Cat# AB1554 RRID:AB_90760) and cytoplasmic (Promega Cat# G3231 RRID:AB_430853) domains of the low-affinity neurotrophin receptor p75^{NTR} (both at 1:400 dilution) were used to identify Schwann cells in vitro (Provenzano et al., 2011; Whitton et al., 2009), determine their specific location within synapse cultures, and their alignment to neighboring spiral ganglion neurons. Western blot and immunocytochemical analysis of antibody specificity

showed the expected lack of protein in p75^{NTR}^{-/-} animals compared to wild type controls (Gehler et al., 2004; Shulga et al., 2012; Wagner et al., 2011).

Anti-Sortilin Antibody—Sortilin is a co-receptor of p75^{NTR} (Lu et al., 2005; Willnow et al., 2008) and was, therefore, utilized to further examine receptor distribution patterns in synapse cultures. The specificity of anti-Sortilin antibody (R&D Systems Cat# AF2934 RRID:AB_2192424, 1:100 dilution) was examined in liver tissue with siRNAs targeted to the SORT1 gene (Musunuru et al., 2010).

Anti-synaptophysin Antibody—Synaptophysin, a presynaptic vesicle protein (Fletcher et al., 1991; Wiedenmann and Franke, 1985), was utilized to examine neuronal process differentiation in synapse cultures. Monoclonal anti-synaptophysin antibody clone SVP-38 (Sigma-Aldrich Cat# S5768 RRID:AB_477523; 1:50 dilution), utilized herein, recognizes a band at its predicted 38kDa molecular weight in Western blot analysis of multiple brain tissues (Gaardsvoll et al., 1988; Morris et al., 2005; Wheeler et al., 2002; Wiedenmann and Franke, 1985). Immunocytochemical evaluation of transfected N2a cells showed that anti-synaptophysin antibody selectively immunolabeled synaptophysin-GFP, while SNAP-25-GFP, GFP, and secondary antibody alone were unlabeled (Flores-Otero et al., 2007).

Image acquisition

Fluorescently labeled images of cochlear sections and isolated spiral ganglion neuronal preparations were acquired with a Hamamatsu 1394 Orca-ER camera attached to a Zeiss Axiovert 200M inverted microscope with deconvolution capability utilizing IPLab Scientific Image Acquisition Software (BD Biosciences). Exposure times and normalization procedures were employed to optimize the images for each experiment. IPLab Scientific Image Analysis Software was utilized to determine neuron number and measure soma size, nuclear size, and process length.

Quantitative analysis

Soma and nuclear size analysis—Soma area and nuclear measurements consisted of outlining each individual cell body and nucleus using IPLab. All neurons in which the soma and nucleus could be unequivocally circumscribed were included in the analysis. To relate the calculated soma area to the relative positions of neurons along the cochlear contour in gangliotopic cultures, a curve was drawn by eye in IPLab, as in Liu et al., 2007. In order to make systematic measurements using immunocytochemical markers, a primary antibody was needed that will both label the full somata size while allowing assessments of the nuclear area for all spiral ganglion neurons. While some antibodies, such as anti-HCN1, specifically label the soma membrane (Fig. 2B, C), they do not label spiral ganglion neurons uniformly (Liu et al., 2014b). Furthermore, nuclear stains such as Hoechst 34580 dye (Sigma-Aldrich) and 4'6'-Diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich), while labeling the entire nucleus, tend to show weaker fluorescence in neurons compared to the surrounding satellite cells (Fig. 2F, G). In contrast, our studies have found anti- β -tubulin antibody labels the full complement of spiral ganglion neurons relatively uniformly throughout the ganglion. By comparing measurements made from anti- β -tubulin antibody to those made from anti-HCN1 antibody for soma size (Fig. 2D) and those made

from Hoechst or DAPI for nuclear size (Fig. 2H) we found that accurate measurements could be made using a single immunolabel for all spiral ganglion neurons.

Survival—While the process of culturing explants prevents unequivocal assessments of the original number of starting neurons to calculate survival, we counted all neurons in each culture dish to get an estimation of surviving neurons in each condition.

Neurite outgrowth—Process outgrowth in synapse cultures was measured from the center of the spiral ganglion explant to the region where axons abruptly changed direction and re-fasciculated in an area we denoted as the ‘Transition Zone’, which is punctuated by anti-synaptophysin labeling (Fig. 1E, arrows, yellow/green). Bifurcation length measurements were made using Image Processing and Analysis in Java Software (ImageJ, NIH) when it was possible to unequivocally trace neurites from individual cell soma to the first branch point.

Statistics—To assess differences in morphometric parameters between anatomical locations or experimental conditions, we used standard procedures described in our previous publications (e.g. (Adamson et al., 2002a; Adamson et al., 2002b; Chen et al., 2011; Flores-Otero and Davis, 2011; Flores-Otero et al., 2007; Liu et al., 2014a; Liu and Davis, 2014b)). Briefly, the soma and nucleus assessments from each tissue explant or cell culture was characterized by numerous individual measurements and the mean of these measurements was counted as an experimental $n=1$. To be complete, as many measurements as possible were made from each explant. Typically a greater number of neurons were available for this analysis in neuronal cultures (average=250), however, an average of 42 and 46 neurons could be measured in individual synapse cultures and postnatal cochlear sections, respectively. The average value was then taken as a quantitative descriptor of each culture or section. The Student’ t -test was used for data comprised of two groups; ANOVA followed by a post-hoc Tukey-Kramer pairwise analysis with a Bonferroni correction was used for multiple group comparisons. Statistical significance was met when $p<0.05$ and is represented by an asterisk; $p<0.01$ is represented by two asterisks. Standard error of the mean (SEM) is indicated by error bars.

Results

The goal of this study was to determine whether the peripheral auditory endorgan, the organ of Corti, regulates the morphological properties of the innervating primary-auditory afferents. We chose to examine two features that vary systematically with the frequency map elaborated within the cochlea: soma size and the PNS/CNS axonal profile.

Soma Size

Tonotopic soma size gradations reported in other species were also observed in postnatal mice and retained in vitro—To take the first step in our examination of spiral ganglion morphology we made neuronal soma measurements from acutely isolated postnatal CBA/CaJ mouse neurons in paraffin-embedded sections. Multiple preparations were evaluated at four different locations: apical, mid-apical, mid-basal and basal regions. The results of this analysis supported the idea that soma size can be related to tonotopic

location, and that basal neurons were significantly larger than apex, mid-apex, and mid-base neurons (Fig. 3A–D, $p < 0.05$). In order to obtain a more complete picture of soma area throughout the trajectory of the ganglion, neuronal soma area was measured in gangliotopic preparations (see Methods) that were maintained for 4 div (Fig. 3E–H). Overall, we found that larger soma areas were obtained from this preparation compared to paraffin-embedded sections. One can account for this size difference due to the tendency for tissues to shrink during the embedding process (Gardella et al., 2003; Quester and Schroder, 1997), the increase in soma size that typically occurs over time in vitro (Zhou et al 2005), and inclusion of somata at sub-maximal cross-sectional areas. Despite the absolute difference in soma size, a very similar pattern was found in gangliotopic cultures when compared to sections. We found that while there was a clear heterogeneity of soma size within each region (gray circles, measurements from a single experiment), mean values from each of five regions averaged for three individual preparations (gray bars) showed a small but graded increase from apex to mid-base regions that culminated in a precipitous increase in the most basal neurons (Fig. 3H). Soma area measurements from neurons isolated from the base were significantly different from apex, mid-apex and middle neuron measurements ($p < 0.05$) this complex organization is consistent with the idea that multiple independent processes likely control soma size along the cochlear contour.

Measurements obtained from the previous two preparations showed clearly that tonotopic differences in soma size were present in vivo and in vitro. Neither preparation, however, was ideal for studying regulation of neuron morphology. To this end, we also examined soma size in neuronal cultures obtained from the extreme apex or base of the ganglion. Evaluation of cell morphology in these reduced cultures was also advantageous for visualizing the shapes and sizes of the neuronal somata (Fig. 4A–H); thus, we determined that both bipolar and pseudomonopolar soma shapes were present (Fig. 4A). With regard to the population of analyzed cells, it is likely that both type I and type II spiral ganglion neurons were included (Berglund and Ryugo, 1987; Kellerhals, 1967; Kiang et al., 1982; Spoendlin, 1971; 1973) although the contribution of the latter is small in our cultures since, like that observed in the ganglion, they represent only 5% of the total number of ganglion neurons (Reid et al., 2004). Occasionally, extremely large neurons with a high cytoplasm to nucleus ratio were observed exclusively in apical cultures (Fig. 4B) fitting the description of the relatively rare type III neurons (Romand and Romand, 1987), which have been shown to innervate outer hair cells (Berglund and Ryugo, 1987). While these neurons were included in our overall analysis, for the purpose of averaging data from each region these cells were excluded because they were well over 3 standard deviations above the mean.

Analysis of separately isolated apical and basal neurons, maintained for 6 div, showed that characteristic soma size differences were indeed retained in vitro. The average of neuronal soma measurements from apical regions (Fig. 4C–E; I) was significantly smaller ($257.7 \pm 5.1 \mu\text{m}^2$, $n=8$) than the average from basal regions ($306.1 \pm 12.0 \mu\text{m}^2$, $n=8$; $p < 0.01$; Fig. 4F–H; I). Nuclear area measurements showed the same trend for apical ($82.0 \pm 2.2 \mu\text{m}^2$, $n=8$) and basal ($91.1 \pm 2.5 \mu\text{m}^2$, $n=8$) neurons ($p < 0.05$; Fig. 4J). We also noted that neuronal cultures, even when isolated from restricted regions of the ganglion, showed heterogeneity (Fig. 4C–E and 4F–H for apical and basal regions, respectively) similar to that observed in gangliotopic cultures (Fig. 3H). Histograms of measurements from a single experiment

comparing the distribution of apical and basal soma sizes are shown in Figure 4K, L, respectively.

Neuronal somata sizes were systematically altered when neuron explants were co-cultured with defined regions of the organ of Corti—Having established that differential soma areas are retained in neuronal cultures, we asked whether they were affected by the presence of the peripheral target tissue. We tested this with synapse cultures (Materials and Methods) by first examining co-cultures of neuron explants and hair cell microisolates from matched tonotopic regions. As shown in Figure 5, size differences were maintained when synapse cultures were evaluated. Apical neurons co-cultured with apical hair cell microisolates had consistently smaller soma areas ($201.5 \pm 4.4 \mu\text{m}^2$, $n=6$; Fig. 5A,C) than those of basal neurons co-cultured with basal hair cell microisolates ($294.6 \pm 8.4 \mu\text{m}^2$, $n=8$; $p<0.01$; Fig. 5B,C). Consistent with our measurements from paraffin-embedded sections and gangliotopic cultures (Fig. 3), middle neurons had soma areas that were intermediate between the apical and basal neuron measurements ($238.5 \pm 12.8 \mu\text{m}^2$, $n=3$; Fig. 5C). While the soma size differences were robust, we also noted heterogeneity comparable to that seen in the other preparations (Fig. 5F, black triangles).

In order to determine whether the peripheral target tissue exerts regulatory effects on neuronal soma area we co-cultured neuronal explants with hair cell microisolates isolated from the opposite tonotopic region. Analysis of these ‘mixed’ synapse cultures showed that neuron soma size takes on the phenotype of the region from which the microisolate originates. Thus, when spiral ganglion explants isolated from the base were paired with microisolates isolated from the apex a significant *decrease* in soma area was found ($201.3 \pm 3.8 \mu\text{m}^2$, $n=10$; $p<0.05$; Fig. 5E,C). To determine whether this decrease was due to compromised survival of the neurons, we counted the number of neurons in each of the synapse cultures. While the original number of neurons in isolated explants undoubtedly varies from preparation to preparation, we found that basal neuron survival in mixed cultures did not decline, but rather increased, when compared to the number of basal neurons surviving in matched cultures (63 ± 6 , $n=10$ vs. 40 ± 1 , $n=8$; respectively, in average neuron number/culture; $p<0.05$). In addition, we found that basal neuron somata showed a tendency to be reduced in size when co-cultured with middle hair cell microisolates ($216.4 \pm 13.4 \mu\text{m}^2$, $n=3$; Fig. 5C).

To test whether the opposite is true, that the smaller apical neuronal somata could be increased in size, we paired them with hair cell microisolates from the base. Neurons in these mixed cultures showed a dramatic enlargement ($295.4 \pm 27.8 \mu\text{m}^2$, $n=9$, $p<0.05$, Fig. 5D, C) compared to apical matched cultures (Fig. 5A), thus, becoming essentially indistinguishable from matched basal synapse cultures (Fig. 5B) without substantial changes to the number of apical neuron surviving (20 ± 7 , $n=9$ vs. 36 ± 4 , $n=6$; for H(B)N(A) and H(A)N(A) respectively, in average neuron number/culture). Furthermore, when individual measurements from apical neurons in a mixed synapse culture were compared to a matched one, the heterogeneity was still manifest (Fig. 5F, gray diamonds). Not unexpectedly, most nucleus area measurements increased linearly with soma area increases (Jorgensen et al., 2007). Two soma measurements, however, differed from this relationship showing a higher cytoplasmic to nucleus area ratio (Fig. 5F, outlined gray diamonds) indicative of the putative

type III neurons (Fig. 4B). Overall, these observations are consistent with the idea that peripheral target tissue can bi-directionally regulate neuron soma area, independent of the neuron's original location.

de novo neurite outgrowth away from microisolates

In addition to soma area, the ratio of the PNS to the CNS axon length also changes systematically along the cochlear contour. Apical neurons *in vivo* further from the Schwann-glia border elaborate longer PNS processes than middle or basal neurons located closer proximity. Thus, the smaller apical neuronal somata elaborate longer PNS axons than the larger basal neuronal somata. To determine whether the same principles underlying soma area also apply to the tonotopic regulation of PNS/CNS axon length ratio, we examined axonal outgrowth *in vitro*.

Spiral ganglion neurite projections in synapse cultures reiterated aspects of the cochlear nerve—In synapse cultures, process outgrowth away from hair cell microisolates produced a stereotypic pattern that was accessible to quantitative assessments. Neurites from spiral ganglion explants projected uniformly away from the co-cultured tissues (Fig. 6A, double arrow) before abruptly altering trajectory at a defined region in the culture dish termed the 'transition zone' (Fig. 6A, double arrowheads). Closer examination of the transition zone revealed that axons bifurcated *below* the transition zone in most synapse cultures examined to date; thus, axon re-fasciculation (Fig. 6B,C), rather than axon branching, was responsible for forming this distinctive region in synapse cultures. Further, the axon lengths measured from individual processes that could be unequivocally traced from the soma to the first bifurcation, while variable, did not show significant differences between apical and basal neurons ($1151.8 \pm 150.5 \mu\text{m}$, $n=6$ vs. $1233.5 \pm 193.7 \mu\text{m}$, $n=5$ for neurons in matched apical and matched basal synapse cultures, respectively). We did observe, however, that axonal bifurcations often contributed to the complex intersecting branching patterns formed well below the transition zone (Fig. 6D). Moreover, while immunolabeling of the presynaptic protein synaptophysin is reduced or absent within axons immediately above the transition zone, there is an abundance of it both at and below the transition zone (Fig. 6A–D). Thus, much like what is observed *in vivo*, where many fibers elaborate pre-synaptic markers after they traverse into the nerve root and beyond (Brown et al., 1988; Fekete et al., 1984; Ryugo, 2008; Ryugo and May, 1993; Ryugo and Rouiller, 1988), one observes aggregated and enlarged puncta possessing the pre-synaptic protein synaptophysin immediately after process trajectory changes. Although precise patterns differ from preparation to preparation, a synaptophysin 'necklace' was frequently observed along the transition zone (Fig. 1E, arrows, Fig. 6A, between double arrowheads, Fig. 6B, arrowheads) below which are small synaptophysin-labeled puncta (Fig. 6C,D).

This organized axonal outgrowth pattern routinely observed in synapse cultures clearly differs from the apparently random process radiations reported for neuron cultures without the peripheral target tissue (Aletsee et al., 2001; Barclay et al., 2011; Staecker et al., 1995). This indicates that co-culturing neurons with hair cell microisolates not only regulates neuronal somata morphology as outlined in the section above, but also appears to orchestrate the directionality and pattern of *de novo* neurite outgrowth. We next compared the synapse

cultures to isolated intact tissue explants in which the spiral ganglion retained both their peripheral and central connections with the organ of Corti and cochlear nucleus, respectively. These tissue explants were maintained in vitro for 2–4 div to eliminate efferent projections and were then subsequently immunolabeled to examine axon bundling from clusters of spiral ganglion neurons. Interestingly, the fiber bundles elaborated *de novo* in vitro were strikingly similar to those observed in these more intact tissues. As can be seen in in Figure 6E, axonal bundles emanating from spiral ganglion somata clusters in a middle explant connected to its peripheral and central targets, like the synapse cultures, extended for a defined distance before changing trajectory (Fig. 6F, arrows). This change in trajectory occurs well above the CNS tissue (Fig. 6E, dotted box) that contains multipolar neuronal profiles (Fig. 6G, H) distinct from the simple bipolar and pseudomonopolar spiral ganglion profiles.

Satellite cells

Putative p75^{NTR}-immunolabeled Schwann cells show differential alignment above and below the transition zone—Anatomically, the parent axon in vivo that extends proximally from each spiral ganglion neuron is actually composed of two separate regions. The first, located in the PNS, is part of the *intracochlear axon* (Fekete et al., 1984), which winds along the cochlear modiolus until it reaches the IAM. The second region, termed the auditory *nerve root*, is part of the CNS beginning at the Schwann-glia border at the IAM, and extending into the cochlear nucleus (Fekete et al., 1984). These two regions, therefore, are myelinated separately by Schwann cells and oligodendrocytes, a distinction that with appropriate structural references becomes quite important when attempting to determine the extent of influence that the hair-cell microisolate has on spiral ganglion neuron morphology. Thus, in order to add aspects of these divisions into our analysis, we sought to label satellite cells that play a role in distinguishing the PNS and CNS regions of the spiral ganglion neuron axons.

The obvious candidates to evaluate are Schwann cells, since the tissues present in our synapse cultures are both peripherally located. We utilized anti-p75^{NTR} antibody to label non-myelinating Schwann cells, since this low-affinity neurotrophin receptor has been localized to them (Provenzano et al., 2011; Whitlon et al., 2009) and absent from spiral ganglion neurons (Tan and Shepherd, 2006). In synapse cultures, anti-p75^{NTR} antibody labeled a large population of cells surrounding the neuron explant and below the transition zone (Fig. 7A, green). The paucity of anti-p75^{NTR} antibody labeled cells between these two regions is a characteristic feature of synapse cultures. A closer look shows that the neuronal somata (Fig. 7B, red) and axonal processes (Fig. 7D, red) appear to be enveloped by anti-p75^{NTR} immunolabel (Fig. 7C, D; green), which highlights regions in which loose and compact myelin typically enwraps the spiral ganglion somata and axons, respectively (Rosenbluth, 1962; Toesca, 1996).

This close alignment, however, only occurs above the transition zone (compare Figures 7E and 7G). Thus, there is a clear distinction at and below the transition zone where we observe that the non-myelinating Schwann cells, like the axons, change their orientation (Fig. 7F) while losing their apparent association with one another (Fig. 7G). This is a particularly

interesting finding because it suggests that neurites elaborated *de novo* from spiral ganglion neurons can display a differential profile depending upon whether they are above or below the transition zone even though they are cultured exclusively with peripheral tissue.

One might expect that if the distinct alignment patterns of anti-p75^{NTR} immunolabeled cells represent an initial step in the process of myelin formation by resident Schwann cells, then myelin-specific protein labeling may also be limited to regions above the transition zone in synapse cultures. In order to test this hypothesis we labeled synapse cultures with anti-myelin basic protein (MBP) antibody, which is capable of recognizing both peripheral and central myelin (Boggs, 2006; Toesca, 1996). The cultures used for this purpose were somewhat older (22 div instead of 17 div) to allow more time for myelination. Consistent with our prediction, while the amount of anti-MBP antibody immunostaining along axons was incomplete (Fig. 8A, green) the segmented pattern of immunolabeling was restricted to regions above the transition zone (Fig. 8).

Because the neurites projecting toward the microisolates have a more defined PNS-like phenotype, we compared anti-MBP immunostaining in processes extending toward and away from the peripheral target tissue. Interestingly, we found that despite the close proximity of the microisolate to the neuron explant the anti-MBP label abruptly terminated on the fibers (Fig. 8B, green, arrowheads) in a region close to the inner hair cells (Fig. 8B, blue) where underlying satellite cells show apparent anti-MBP labeling (Fig. 8B, green, asterisk). Not all axons are myelinated and occasionally node-like interruptions were noted along the fibers, however, close to the neuronal somata the termination of anti-MBP immunolabeling was generally not apparent (Fig. 8C). In comparison, we noted that anti-MBP antibody labeling also abruptly terminated as fibers approached the transition zone (Fig. 8E). Whether this is orchestrated by the altered Schwann cell alignment noted above cannot be unequivocally determined at this time because we do not know whether anti-MBP staining would breach the transition zone should tissues be maintained for longer times in vitro. This issue may be partially addressed, however, by the atypical positioning of some neuronal somata in the example shown in Figure 8. Rarely, if ever, do we observe neurons themselves at the transition zone, yet, in this unusual example (Fig. 8A, lower left box) we noted an anti-MBP immunolabeled process extending from one of these cells toward the putative PNS side of the transition zone (Fig. 8D, arrows). Processes of the same neuron and others extending into the putative CNS side, by contrast, were unlabeled. While we do not yet know whether this pattern is retained in even older cultures (>22 div), it reveals a heretofore unexpected specification of axons under conditions in which the CNS environment is missing.

Sortilin-labeled satellite cells were localized to regions above the transition zone—To explore further the nature of the difference between regions above and below the transition zone in synapse cultures, we probed the distribution of a p75^{NTR} co-receptor using an anti-Sortilin antibody. While we had initially expected anti-Sortilin to co-label p75^{NTR}-expressing cells (Lu et al., 2005; Willnow et al., 2008), we instead found that in addition to labeling the neuronal somata (Fig. 9B), the antibody also labeled an altogether different class of satellite cell with a wide in vitro distribution (Fig. 9A, green), that was distinctly different from the p75^{NTR}-immunolabeled putative Schwann cells (Fig. 9C, red). The

Sortilin-immunolabeled cells (green) were found within the hair cell microisolate (Fig. 9A, dotted shape) and extended to the transition zone (Fig. 9A, double arrowheads) where they terminated abruptly, forming a discrete border (Fig. 10). Thus, the Sortilin-immunolabeled cells appeared to form a carpet beneath neuron processes aligned with p75^{NTR}-labeled Schwann cells (Fig. 10A–D) that ended at the transition zone (Fig. 10E–H) and was essentially missing well below it (Fig. 10 I–L). The abrupt change in Sortilin immunolabeling does not indicate compartmentalization of all satellite cells above and below the transition zone, however. As can be observed from anti-p75^{NTR} immunolabeling (Fig. 10E) and DAPI-labeled nuclei (Fig. 11, blue), satellite cells are distributed across the Sortilin-labeled interface of the transition zone. Thus, Sortilin immunolabeling patterns serve to highlight another specialization formed in vitro that further defines differences between the cellular features reiterated above and below the transition zone.

Tonotopic Axon length

From the experiments described above, it appears that the *de novo* processes elaborated from spiral ganglion explants as well as the surrounding satellite cells are specialized within defined regions of synapse cultures. Interestingly some of these features reiterate the in vivo organization that distinguishes PNS from CNS. Uniform directionality, alignment of non-myelinating Schwann cells, preferential MBP-immunolabeled segments, lack of branching and synaptophysin localization are all consistent with a peripheral axonal phenotype. Thus, if the neurite bundles above the transition zone reflect the features of intracochlear axons, one might expect that they should extend for longer distances in apical matched synapse cultures compared to basal ones. Consistent with this hypothesis we found that when cultures were assessed at the same div without added neurotrophins processes extending from apical matched co-cultures (Fig. 12A,D) had a longer organized trajectory ($984.8 \pm 92.9 \mu\text{m}$, $n=7$) than axons extending from middle ($740.3 \pm 105.4 \mu\text{m}$, $n=5$; Fig. 12B,D) which was significantly different from basal matched co-cultures ($344.9 \pm 50.1 \mu\text{m}$, $n=9$; $p<0.01$; Fig. 12C,D). Thus the prominent axonal trajectory organization evident from spiral ganglion neuron explants co-cultured with organ of Corti microisolates followed the relative tonotopic length differences predicted by intracochlear axon lengths measured from cat in vivo (Lieberman and Oliver, 1984), albeit with different absolute values.

To determine whether organ of Corti microisolate location was responsible for the differences in axon length, we evaluated the same parameter in mixed co-cultures in which the microisolate and neuron explants were taken from different regions of the cochlea. We found that the microisolate location did have a significant impact. The organized axon trajectory of basal spiral ganglion explants co-cultured with apical microisolates ($911.8 \pm 41.7 \mu\text{m}$, $n=10$) were not different from matched apex co-cultures and were significantly longer than the matched base co-cultures ($p<0.05$). Similarly, basal neurons paired with middle microisolates ($610.2 \pm 113.2 \mu\text{m}$, $n=4$) were not distinguishable from middle matched co-cultures (Fig. 12D). Apical neurons co-cultured with basal microisolates ($508 \pm 63.4 \mu\text{m}$, $n=10$) were not different from matched basal co-cultures and were significantly shorter than the matched apical co-cultures ($p<0.05$). Thus, apical microisolates promoted longer axonal trajectories, whereas the basal microisolates were responsible for significantly shorter ones. Similar to the regulation of neuronal soma area, therefore, these observations

are consistent with the idea that the peripheral targets orchestrate tonotopically-relevant morphological phenotypes of spiral ganglion neurons.

Discussion

Nowhere in an organism is there such exquisite morphological diversity as that demonstrated by the wide range of unique neuronal shapes and sizes throughout the brain. From the elaborate fan-shaped dendrites of cerebellar Purkinje cells to the small, simple retinal bipolar neurons, the parameters of size and shape generally have a clear and logical relationship to one another. While one might expect that neurons with elaborate arbors and long axon projections require metabolic support from larger somata and that smaller somata sustain simpler cells, this is not always strictly the case (Sun et al., 2002). The complexity in the spiral ganglion, for example, is due to the tonotopically-graded soma size of neurons that have similar innervation patterns. All type I neurons make one-to-one synaptic connections to inner hair cells and support the same parent axon length. Nonetheless, there is an inverse relationship between soma size and the synaptic area of the end bulb of Held, which is the largest of the multiple presynaptic specializations made by spiral ganglion neurons. Overall, neurons that display the broadest synaptic regions along their bushy cell targets in the cochlear nucleus originate from the apical spiral ganglion where the somata are the smallest (Rouiller et al., 1986). There are also subtle morphological differences related to threshold and spontaneous firing rate of spiral ganglion neurons (Sento and Ryugo, 1989) superimposed on these morphological patterns that may contribute to the local heterogeneity prominently displayed in these cells.

Soma size

The close correspondence of soma size to tonotopic location and its reverse relationship to end bulb of Held presynaptic complexities suggests that this parameter may be intimately controlled by the peripheral target tissue. One test of this regulation was to determine whether neuronal somata size could be either increased or decreased, independent of survival in vitro. While many studies report that soma area changes with differing manipulations (Agterberg et al., 2008; Leake et al., 2013; Shepherd et al., 2005), this study shows that control can be bidirectional. These observations support the hypothesis that the peripheral target tissue possesses the capability to orchestrate fine details of a fundamental morphological parameter. This may not be unexpected considering that the absolute size of the soma in combination with other factors, such as myelination and ion channel composition, is critical for assuring conduction across a region in which action potentials are vulnerable due the interposition of the soma in the conduction pathway, similar to branch point failure (Debanne et al., 2011; Foust et al., 2010; Gemes et al., 2013; Zhou and Chiu, 2001).

PNS/CNS length ratio

Another prominent tonotopically-regulated morphological feature of the spiral ganglion is the PNS to CNS axon ratio. To study this feature under controlled conditions in vitro, however, requires that PNS and CNS axons can be distinguished from one another. Remarkably, synapse cultures recapitulate many of the well-characterized features of the in

vivo innervation patterns, yet without the presence of central synaptic targets or tissues (Fig. 13). Contrary to typical spiral ganglion neuron cultures in which process outgrowth is disorganized and unpatterned (Aletsee et al., 2001; Barclay et al., 2011; Staecker et al., 1995), the organization of the axon trajectory in synapse cultures away from the peripheral target tissue is striking.

When viewing these cultures, a prominent feature was the presence of a transition zone, characterized by the re-bundling of axons (not depicted in Fig. 13). When individual fibers could be traced unequivocally through the transition zone to the first bifurcation point, we found that apical and basal parent axons did not differ significantly in length (Fig. 13, dotted line). This finding was consistent with measurements made from cat spiral ganglion neurons *in vivo*, (Lieberman and Oliver, 1984), although the absolute values are expectedly smaller in mouse tissues. Moreover, because apical axons elaborated a greater proportion of their length before the transition zone, it follows that higher PNS to CNS length ratios (Fig. 13, dashed line) were also reiterated in synapse cultures.

Presynaptic specializations

It is not only the length and re-bundling of the *de novo* axon outgrowth that distinguishes PNS-like profiles above the transition zone from the CNS-like profiles below it. We also observed that the elaboration of synaptophysin differs between each area (Fig. 13, yellow symbols). As predicted, very little labeling for this presynaptic, vesicle-associated protein was observed within the region of uniformly projecting PNS-like axon profiles. On the other hand, a profusion of synaptophysin-labeled puncta was observed in the main axons and small processes that branched from them in the region of the transition zone. These observations appear to reproduce the small processes terminating in synaptic-like puncta that have been found in spiral ganglion axons *in vivo* once they cross the Schwann-glia border and extend into the nerve root (Brown et al., 1988; Fekete et al., 1984; Ryugo, 2008; Ryugo and May, 1993; Ryugo and Rouiller, 1988).

Myelination

Apart from the morphological distinctions characterized for spiral ganglion neuron PNS and CNS axons, a prominent feature for any nerve that crosses the Schwann-glia border is its distinctive myelination (Sherman and Brophy, 2005). Axon characteristics above and below the transition zone in synapse cultures also differ in this regard. The most obvious feature was the differential alignment of satellite cells with axonal processes elaborated from both sides of the neuronal soma. The satellite cells possessed many of the hallmarks of non-myelinating Schwann cells due to their small size, bipolar morphology, and p75^{NTR} immunolabeling (Chen et al., 2011; Salzer and Bunge, 1980; Whitlon et al., 2010; Whitlon et al., 2009). In some cases these satellite cells enwrapped axonal segments, commensurate with the observation that MBP-immunolabeled segments surrounded axons but only above the transition zone. Thus, despite the fact that resident Schwann cells had access to all axons in the culture dish, only the axons above the transition zone showed features characteristic of PNS neurons.

Satellite cells

How does the organ of Corti orchestrate these distinctive axonal phenotypes? To begin to address this complex question, we present evidence that cells originating in the cochlea and resident within hair cell microisolates may play a role. In order to build upon our findings with anti-p75^{NTR}, we chose to evaluate the distribution of Sortilin, a potential p75^{NTR} binding partner. Our studies showed that multiple distinct cell types were Sortilin-immunolabeled in synapse cultures including supporting cells within the organ of Corti, migrating epithelial-like cells, and spiral ganglion neurons. This is consistent with Sortilin distributions in the inner ear of postnatal rats. Epithelial cells lining the cochlear duct (marginal, Dieter's, Hensen and Reissner's membrane cells), as well as spiral ganglion neurons (Tauris et al., 2011) and their surrounding Schwann cells (Provenzano et al., 2011) were all labeled with anti-Sortilin antibody.

Interestingly, we found that labeling with anti-Sortilin antibody in synapse cultures did not co-localize with p75^{NTR}-stained, non-myelinating Schwann cells. Instead, anti-Sortilin antibody labeled a different class of satellite cell. Thus, this organization rules out an apoptotic role for the receptor pair in the presence of pro-neurotrophins (Nykjaer et al., 2004) since Sortilin and p75^{NTR} were not co-localized in our system. Moreover there did not appear to be fewer cells at or above the transition zone. Nevertheless, it was clear that the carpet of anti-Sortilin immunolabeled satellite cells extending from the hair cell microisolates abruptly ended at the transition zone, showing yet another distinguishing feature of this region.

While we do not know the identity of the Sortilin immunolabeled cells in synapse cultures, it has been shown that Sortilin is present in a wide range of cell types (Kalous et al., 2012; Martin et al., 2003; Provenzano et al., 2011; Tauris et al., 2011). In the auditory periphery, it is tempting to speculate the Sortilin positive cells may be the very same otic mesenchyme that regulate the distinct bundling patterns of spiral ganglion distal axons via ephrin-B2/EphA4 interactions (Coate et al., 2012). However, the relatively low levels and uniform distribution of Sortilin immunolabeled cells in synapse cultures underlying the axons in the region above the transition zone serves as an initial indicator that these cells are either distinct from otic mesenchyme or serve in a different capacity for proximal axons. Moreover, the multifunctional nature of Sortilin suggests additional types of regulation. For example, the central function of Sortilin as a sorting receptor directing target proteins could contribute to regulated release of multiple elements (Petersen et al., 1997; Willnow et al., 2008) including growth factors such as BDNF (Chen et al., 2005). On the other hand, neurotensin binding to Sortilin and subsequent regulation of cell migration via phosphatidylinositol 3-kinase (PI-3-kinase) and mitogen-activated protein (MAP) kinase pathways (Martin et al., 2003) could underlie the differential distances noted between the transition zone and hair cell isolates. These and other functional contributions of Sortilin and p75^{NTR} will be systematically explored in subsequent studies to determine whether these receptors play a role in regulating the PNS/CNS outgrowth patterns in synapse cultures.

An address in the time domain

While it is clear that the vast array of morphological features elaborated by neurons throughout the nervous system are tailored to their functional requirements and restrictions, the extent of this control is revealed in the exquisite organization of the spiral ganglion. The tonotopic regulation of soma area and ratio of PNS / CNS axon length by the peripheral endorgan, while perhaps surprising, makes sense. Once frequency is established as a unique location within the cochlea, these tonotopic specializations are reiterated, rather than reinvented, at the next level of processing. Thus, location-specific morphological information representing the *time* domain is sequentially mapped and, thus, traverses the boundary from the sensory endorgan to the signaling neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Both authors had access to all data in the study and verify its accuracy and integrity. Data acquisition (FLS), Analysis and interpretation (FLS, RLD), Drafting of the manuscript (RLD), Critical revision of the manuscript (FLS, RLD), Statistical analysis (FLS, RLD), Obtained funding (RLD), Administrative, technical and material support (RLD), Study supervision (RLD).

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Figure 1.

Synapse Cultures are co-cultures of organ of Corti microisolates paired with spiral ganglion neuron explants maintained in vitro for up to 22 days. **A**, A subset of spiral ganglion neurons are fluorescent in a Thy1-YFP (12) P6 wholemount preparation (label appears green due to FITC filter settings). When utilized for a synapse culture, the hair cell microisolate apex [H(A), dotted line] is removed separately from the neuron explant [N(A), arrows]. **B**, A matched synapse culture composed of an apical microisolate (dotted line) closely positioned adjacent to an apical neuronal explant (arrows) in the correct orientation (peripheral processes toward the microisolate) at the time of plating. While this is an example of a ‘matched’ culture, disparate regions were also plated next to one another to construct ‘mixed’ cultures. The asterisk indicates a scratch mark on the culture dish surface. **C**, A matched Thy1-YFP (16) apical synapse culture showing processes that extend toward the microisolate (upper left) and away from it (lower right). YFP expression (green) is retained in some but not all neurons after 12 div. Although many of the neurons do not successfully regenerate to the peripheral tissue, others form either the elongated branches that typify type II processes (arrows) or more tightly branched profiles that typify type I processes early postnatally (asterisks). **D**, Brightfield view of a hair cell microisolate (dotted line), placed adjacent to a neuronal explant (arrowheads) at time of plating to form a matched apex synapse culture supplemented with 5ng/ml NT-3. The double-headed arrow indicates the distance from the center to the edge of the explant (192.6 μm). The asterisk indicates an area

of scratch marks on the culture dish surface. **E**, Same culture as in (D), now 17 div, labeled with anti- β -tubulin (red), anti-synaptophysin (green), and anti-calbindin (blue) antibodies. The double-headed arrow (974.7 μ m) indicates the distance from the center of the explant (SGN) to the transition zone (arrows) where fibers abruptly change trajectory and elaborate synaptophysin puncta (green/yellow). The processes grow out for greater distances beyond this point (not shown).

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Figure 2.

Both somata and nuclear area were accurately measured with anti- β -tubulin antibody. **A-D**, Anti- β -tubulin antibody measurements correspond to those taken from anti-HCN1 antibody that outlines the neuronal membrane. **A-B**, Spiral ganglion neurons from P6 CBA/CaJ mice labeled after 7 div with **(A)** anti- β -tubulin antibody (red) and **(B)** anti-HCN1 antibody (green). **C**, Merged image, of panels A and B, white and orange dashed lines represent soma area measurements made from the anti- β -tubulin and anti-HCN1 antibody stained neuron, respectively. **D**, Scatter plot of soma area measurements made from the anti- β -tubulin and anti-HCN1 antibodies in a single experiment (P6, 7 div) shows a close correlation. **E-H**, Anti- β -tubulin antibody measurements correspond to those taken from Hoechst and DAPI dye that specifically label cell nuclei. **E-F**, Spiral ganglion neurons (CBA/CaJ) labeled with **(E)** anti- β -tubulin (red) and **(F)** Hoechst dye (blue). **G**, Merged image of panels E and F. Arrowheads in F and G demarcate the lighter labeling of neuronal nuclei compared to the nuclei of surrounding satellite cells. **H**, Scatter plot of nuclear area measurements made from anti- β -tubulin and Hoechst or DAPI dye in a four separate experiments show a close correspondence. Black lines in D, H are least-squares linear fits. Scale bar in G applies to A-C and E-G.

Figure 3.

Soma area has a non-linear relationship to tonotopic position. **A-D**, Soma area measurements in fixed tissues taken from CBA/CaJ postnatal spiral ganglion neurons show that neuronal size increases incrementally from apex to mid-base and then becomes substantially larger in the base. **A**, Low magnification image of a paraffin-embedded P5 cochlear section. **B-C**, High magnification images of extreme apical and basal regions in **A**, respectively. **D**, Averaged soma size measurements for distinct tonotopic regions of the P5-7, CBA/CaJ ganglion. Experiment number is shown within the bars and * represents $p < 0.05$, in this and subsequent figures. **E-H**, Soma area measurements made from ganglionic cultures changed non-linearly from the apex to the base. **E**, Low magnification image of a ganglionic culture from a P7 CBA/CaJ mouse, 4 div. **F-G**, High magnification images of apical and basal regions in **E**, respectively. **H**, Scatter plot of soma size measurements taken from the example shown in **E**. Soma area plotted against the percent distance from the apex. Gray symbols represent individual measurements from a single preparation distributed along the ganglionic contour as in Liu et al., 2014, bars are the mean values calculated from five evenly-spaced regions and averaged for each of three different preparations.

Figure 4.

Neuronal cultures contain differently shaped neurons that are distinguished by soma and nuclear size between apical and basal regions. **A**, Anti-MAP2 antibody highlights neuronal morphologies that range from bipolar to pseudomonopolar (P9 CBA/CaJ, 7 div). **B**, Large neurons with small nuclear to cytoplasmic size ratios that fit the profile of type III spiral ganglion neurons are found in apical, but not basal neuronal cultures. Anti- β -tubulin labeled apical neuron from a P6 CBA/CaJ mouse, 6 div. **C–H**, Spiral ganglion neurons labeled with anti- β -tubulin. **C–E**, Apical neurons, while smaller overall than basal neurons, were heterogeneous in size. **F–H**, Basal neurons, while larger overall than apical neurons, also showed soma and nuclear size heterogeneity. **I**, Averaged soma area measurements from 8 experiments were significantly smaller in apical than basal neurons. **J**, Averaged nuclear area measurements from the same 8 experiments in **I** were significantly different. **K–L**,

Histograms from a single experiment of apical (K) and basal (L) soma area measurements.

** represents $p < 0.01$.

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Figure 5.

Hair cell microisolates modulate spiral ganglion neuron soma size. **A-B**, Spiral ganglion neurons in matched apical and basal P6 synapse cultures, respectively, labeled after 16–17 div with anti- β -tubulin. **C**, Averaged soma area measurements from mixed and matched P6 synapse cultures (15–17 div). **D-E**, Spiral ganglion neurons in mixed P6 synapse cultures labeled after 16–17 div with anti- β -tubulin. The normally small apical neurons in matched cultures (**A**) are transformed into large ones when co-cultured with basal microisolates (**D**) while the normally large basal neurons in matched cultures (**B**) are transformed into small ones when paired with apical microisolates (**E**). Scale bar in E applies to all images. **F**, Scatter plot of nuclear vs. soma area for one matched apical H(A)N(A) synapse culture and one mixed H(B)N(A) synapse culture, both isolated from P6 CBA/CaJ cochleae and assessed at 17 div. H, hair cell microisolate; N, neuronal spiral ganglion explant; (A) apical; (M) middle; (B) base.

Figure 6.

Stereotypic outgrowth patterns from spiral ganglion neuron explants in synapse cultures reproduce features observed from more intact preparations. **A–D**, Synapse culture and **E–H**, organ of Corti/spiral ganglion/cochlear nucleus culture labeled with anti-β-tubulin (red), anti-synaptophysin (green), and anti-calbindin (blue) antibodies. **A**, Low magnification montage of a P6, 17 div synapse culture supplemented with 5ng/ml BDNF and 5ng/ml NT-3 to enhance neuronal survival. Neurons (red) elaborate axonal processes that project uniformly away from the SGN explant (double arrow) to the transition zone (double

arrowheads), where the axon trajectory changes abruptly. Dotted curve indicates the location of the microisolate. SGN indicates the region of the spiral ganglion explant where neuronal somata are co-labeled with β -tubulin and synaptophysin (yellow), some of the somata appear to have migrated from the explant toward the transition zone (double arrowheads) on the right side of the preparation. **B**, High magnification image of the transition zone (dotted box in panel A) showing fiber trajectory changes and synaptophysin puncta (green, arrows). **C**, High magnification image of another synapse culture transition zone (double arrowheads) below which is widely dispersed synaptophysin-labeled puncta (green/yellow) amongst the neuronal processes (red; 5ng/ml BDNF, 17 div, P6). **D**, High magnification image below the transition zone (box in panel A) shows elaborate fiber aggregates and associated synaptophysin puncta (green/yellow). **E**, Low magnification montage of a culture in which the spiral ganglion was removed while still attached to the organ of Corti and cochlear nucleus target tissues. Tissue was maintained for 4 div in order to eliminate efferent fibers that originate from higher brainstem regions. **F**, High magnification of axon bundles (box in panel E); arrows indicate a change in axon trajectory. **G–H**, Examples of anti- β -tubulin labeled neuronal profiles (red) in the cochlear nucleus region of the preparation (dotted box in panel E) that have distinctly different shapes from the bipolar and pseudomonopolar soma morphology that typifies spiral ganglion neurons. These neuronal profiles are often surrounded by anti-synaptophysin antibody labeled puncta (green/yellow). Scale bar in H applies to G,H.

Figure 7.

Satellite cells displaying non-myelinating Schwann cell characteristics are present in synapse cultures. **A–G**, Matched apex synapse culture labeled with anti- β -tubulin antibody (red) and anti-p75^{NTR} antibody against the cytoplasmic domain (green). **A**, Low magnification montage of a P6 matched apical synapse culture, 15 div. The location of the transition zone is indicated with double arrowheads. Dotted curve and SGN indicate the location of the microisolate and spiral ganglion explant, respectively. **B–D**, high magnification images from a different matched apex P6 synapse culture at 17 div. **B–C**,

High magnification images of anti-p75^{NTR} immunolabeled spiral ganglion neuronal somata (green) with (B) and without (C) anti- β -tubulin label (red). Scale bar in C applies to panels B and C. **D**, A single neuronal process (red) surrounded by a non-myelinating Schwann cell-like profile (green); arrow indicates its putative nucleus. **E**, Alignment of neuronal processes (red) and non-myelinating Schwann cell-like profiles (green) above the transition zone (upper box in panel A). **F**, Change of neuronal process (red) and non-myelinating Schwann cell-like (green) trajectory at the transition zone (middle box in panel A). **G**, Non-aligned neuronal processes (red) and non-myelinating Schwann cell-like profiles (green) below the transition zone (lower box in panel A). A magenta-green version of this figure is available online as Supporting Information.

Figure 8.

Myelin basic protein immunolabeled segments surrounding neuronal processes above but not below the transition zone. **A–E**, Synapse culture labeled with anti- β -tubulin (red), anti-MBP antibodies (green), and calbindin (blue). **A**, Low magnification montage of a P6 matched base synapse culture, 22 div. Dotted line indicates the general position of the hair cell microisolate, double arrowhead indicates the location of the transition zone. The solid arrow indicates neuronal somata that are situated progressively closer to the transition zone. **B**, High magnification image of processes extending toward the hair cell microisolate (upper box, panel A) highlights the abrupt termination of MBP-immunolabeling below the hair cell region. Arrows indicate pillar-like cell profiles labeled with anti- β -tubulin antibody (red); arrowheads indicate anti-MBP termination; asterisk indicates light anti-MBP labeling of underlying satellite cells. **C**, High magnification image of processes projecting through the spiral ganglion explant (mid-left box, panel A). **D**, High magnification image of cell soma (arrow, nucleus not in focus) that apparently migrated to the transition zone (lower box,

panel A). Note that anti-MBP-immunolabeling is only present in the process extending above the transition zone (arrows). **E**, High magnification image of one myelinated fiber amongst several approaching the transition zone (mid-right box, panel A). Asterisk indicates the upper extent of the transition zone. A magenta-green version of this figure is available online as Supporting Information.

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Figure 9.

An additional class of satellite cells identified with anti-Sortilin antibody underlie the tissues elaborated above the transition zone. **A–C**, Synapse culture labeled with anti-Sortilin (green) and anti-β-tubulin (blue) antibodies and anti-p75^{NTR} antibody against the extracellular domain (red). **A**, Low magnification montage of a P6 matched apex synapse culture at 15 div supplemented with 5ng/ml NT-3. Dotted line indicates the placement of the hair cell microisolate; anti-Sortilin immunolabeling was noted in this region (green). **B**, High magnification image of spiral ganglion neuron somata within the explant (top box, panel A).

Note the punctate anti-Sortilin immunolabeling within the neuronal somata (blue-green) surrounded by a halo of anti-p75^{NTR} immunolabeling (arrows, red). **C**, High magnification image of a Schwann cell-like profile (red) surrounding a nerve fiber (blue) above the punctate labeling of Sortilin-immunolabeled satellite cells (green) localized superior to the transition zone (lower box, panel A). Arrow indicates the putative cell soma of the Schwann cell profile. A magenta-green version of this figure is available online as Supporting Information.

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Figure 10.

Sortilin immunolabeling is preferentially localized to satellite cells that reside beneath the PNS-like axonal domain and borders the transition zone. **A–L**, Regions from the synapse culture shown in Figure 9 above (A–D), at (E–H) and below (I–L) the transition zone labeled with anti-Sortilin (green) and anti-β-tubulin (blue) antibodies, and anti-p75^{NTR} against the extracellular domain (red). **A–C**, The highly organized Schwann cell-like profiles (A) above a carpet of Sortilin immunolabeled cells (B) show the same trajectory as the neuronal processes (C). **D**, Merged image of A–C. Scale bar applies to panels A–D. **E–G**, The transition zone is demarcated by reorganization of Schwann cell-like profiles (E), the abrupt delimitation of Sortilin immunolabeled cells (F) and the change of neuronal process trajectory (G). **H**, Merged image of E–G. Scale bar applies to panels E–G. **I–K**, Further below the transition zone non-myelinating Schwann cell-like profiles are reduced and not aligned to neurons (I), Sortilin-labeling is essentially absent (J), and neuronal fibers elaborate complex patterns (K). **L**, Merged image of I–K. Scale bar applies to panels I–L. A magenta-green version of this figure is available online as Supporting Information.

Figure 11.

Not all satellite cells are preferentially distributed at the transition zone. **A–C**, Synapse culture labeled with anti-Sortilin (green) and anti- β -tubulin (red) antibodies and stained with diamidino-2-phenylindole (DAPI) dye to stain DNA (blue). **A**, Low magnification montage of a P6 matched middle synapse culture at 17 div. Dotted line indicates the placement of the hair cell microisolate; SC indicates elongated satellite cells with abundant β -tubulin that likely are pillar cells. DAPI staining was deficient in the areas highlighted by asterisks, yet relatively uniform across the transition zone (arrow heads). **B–C**, High magnification image of transition zone (box, panel A). **B**, Neurites labeled with β -tubulin (red) show a change in trajectory as they cross the transition zone, which is highlighted by the border of Sortilin-labeled cells (green). **C**, DAPI staining alone (blue) shows no indication of cellular distribution patterns specifically associated with the transition zone. Similar observations were made from apex and base matched cultures.

Figure 12.

The length of the organized spiral ganglion processes elaborated *de novo* from neuron explants to the transition zone is modulated by hair cell microisolates. **A–C**, Matched apical (A), middle (B) and basal (C) synapse cultures labeled with anti-β-tubulin (red) and anti-calbindin (blue) antibodies (P6, 15–17 div for all examples). Scale bar in panel C applies to A–C. Double headed arrows indicate the transition zone for panels A–C. **D**, Process length in matched and mixed synapse cultures shows a tonotopic gradient regulated by hair cell microisolates. The distance from the mid-region of the spiral ganglion explant to the transition zone was longest in synapse cultures in which neurons were co-cultured with apex microisolates compared to those co-cultured with basal microisolates. A magenta-green version of this figure is available online as Supporting Information.

Figure 13.

Location-dependent signals resident in synapse cultures set the ‘address’ of neurons within the spiral ganglion, allowing them to elaborate the appropriate tonotopically-associated soma size and PNS / CNS axonal ratios. The PNS-like axonal phenotype above the transition zone is typified by 1) longer axon bundles in the apex compared to the base, 2) alignment of p75^{NTR}-labeled non-myelinating Schwann cells, 3) anti-myelin basic protein antibody labeling, 4) exclusion of synaptophysin, 5) limited or no axon branching. The Transition Zone is characterized by an abrupt change of axon trajectory (not shown) and

non-myelinating Schwann cell orientation. The CNS-like phenotype below the transition zone is characterized by 1) elaboration of synaptophysin puncta, 2) non-alignment of p75^{NTR}-labeled cells. 3) lack of myelination, 4) the presence of axon branching. A magenta-green version of this figure is available online as Supporting Information.

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Table 1

List of Primary Antibodies

Antibody	Immunogen	Type, Company, Cat#, RRID	Dilution
Calbindin	Chicken calbindin D-28k	Mouse monoclonal antibody (Swant Cat# 300 RRID:AB_10000347)	1:100
Calbindin	Recombinant rat calbindin D-28k	Polyclonal antibody raised in rabbit (Swant Cat# CB 38 RRID:AB_10000340)	1:100
class III β -tubulin (TUJ1)	Microtubules from rat brain	Mouse monoclonal antibody (Covance Research Products Inc Cat# MMS-435P-100 RRID:AB_663338)	1:350
class III β -tubulin	Generated against the TUJ1 epitope	Polyclonal antibody raised in rabbit (Covance Research Products Inc Cat# PRB- 435P-100 RRID:AB_10063850)	1:200
HCN1	Intracellular N-terminus (amino acids 6- 24) from rat hyperpolarization-activated cyclic nucleotide-gated channel 1	Polyclonal antibody raised in rabbit (Alomone Labs Cat# APC-056 RRID:AB_2039900)	1:200
MAP-2	Microtubule-associated protein from rat brain	Polyclonal antibody raised in rabbit (EMD Millipore Cat# AB5622 RRID:AB_91939)	1:100
MBP	Human myelin basic protein from brain	Polyclonal antibody raised in rabbit (Millipore Cat# AB980 RRID:AB_92396)	1:100
p75 ^{NTR}	Extracellular fragment from the third exon (amino acids 43–161) of mouse p75 neurotrophin receptor	Polyclonal antibody raised in rabbit (Millipore Cat# AB1554 RRID:AB_90760)	1:400
p75 ^{NTR}	Cytoplasmic domain of human p75 neurotrophin receptor	Polyclonal antibody raised in Rabbit Promega Cat# G3231 RRID:AB_430853	1:400
Sortilin	Mouse myeloma cell line NS0-derived recombinant mouse Sortilin	Polyclonal antibody raised in Goat (R&D Systems Cat# AF2934 RRID:AB_2192424)	1:100
Synaptophysin	Rat retina synaptosome, clone SVP-38	Mouse monoclonal antibody (Sigma-Aldrich Cat# S5768 RRID:AB_477523)	1:50