Research Article

The Structural Development of the Mouse Dorsal Cochlear Nucleus

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ABSTRACT

The dorsal cochlear nucleus (DCN) is a major subdivision of the mammalian cochlear nucleus (CN) that is thought to be involved in sound localization in the vertical plane and in feature extraction of sound stimuli. The main principal cell type (pyramidal cells) integrates auditory and non-auditory inputs, which are considered to be important in performing sound localization tasks. This study aimed to investigate the histological development of the CD-1 mouse DCN, focussing on the postnatal period spanning the onset of hearing (P12). Fluorescent Nissl staining revealed that the three layers of the DCN were identifiable as early as P6 with subsequent expansion of all layers with age. Significant increases in the size of pyramidal and cartwheel cells were observed between birth and P12. Immunohistochemistry showed substantial changes in synaptic distribution during the first two postnatal weeks with subsequent maturation of the presumed mossy fibre terminals. In addition, GFAP immunolabelling identified several glial cell types in the DCN including the observation of putative tanycytes for the first time. Each glial cell type had specific

spatial and temporal patterns of maturation with apparent rapid development during the first two postnatal weeks but little change thereafter. The rapid maturation of the structural organization and DCN components prior to the onset of hearing possibly reflects an influence from spontaneous activity originating in the cochlea/auditory nerve. Further refinement of these connections and development of the non-auditory connections may result from the arrival of acoustic input and experience dependent mechanisms.

Keywords: cochlear nucleus, dorsal cochlear nucleus, auditory brainstem, postnatal development, mouse

INTRODUCTION

The mammalian cochlear nucleus (CN) consisting of the dorsal and ventral subdivisions (DCN and VCN, respectively) receives direct input from the cochlea. The role of the DCN in auditory processing is not very well established but is thought to include sound localization in the vertical plane and identification of the sound source by extracting spectral features of sound and integrating with other sensory inputs (May 2000; Shore and Zhou 2006; Koehler et al. 2011; Koehler and Shore 2013). In addition, the DCN may

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be an important nucleus for processing essential speech cues: speech processing deficits such as difficulties extracting speech in background noise may involve DCN abnormalities (Caspary et al. 2005; Schatteman et al. 2008; Walton 2010). Abnormalities in the DCN have also been implicated in development of tinnitus possibly associated with hyperactivity of the principal cells due to reduced inhibition as well as increased excitation from the non-auditory circuitry following cochlear injury (Wang et al. 2009; Middleton et al. 2011; Dehmel et al. 2012; Koehler and Shore 2013). In children, disorders of neural processing in the DCN could be associated with abnormal development of the CN or injury at birth, for example from perinatal hypoxia, sound deprivation or abnormal hormonal levels (Webster 1983; Harris and Rubel 2006; McClure et al. 2006; Luoma and Zirpel 2008).

The DCN of the mammalian auditory system is a layered structure resembling the cerebellum that sits in the dorsolateral brainstem beneath the floor of the fourth ventricle (Cant 1992; Young and Oertel 2004). A lamina of small cells intermixed with granule cells separates the DCN from the VCN with additional granule cells forming the outer shell of the VCN. Three well-defined neuronal layers are found within the adult DCN: the deep layer, the pyramidal (sometimes referred to as the fusiform) cell layer and the more superficial molecular layer. The deep layer contains one of the principal cell types, the giant cells, vertical cells (an inhibitory interneuron) and neuronal processes including the basal dendrites of pyramidal (or fusiform) cells and the incoming auditory nerve fibres (ANFs). Granule cells and Golgi cells can also be found in the deep layer (Mugnaini et al. 1980a). The pyramidal cell layer contains the cell bodies of the major principal cell type (pyramidal cells) as well as Golgi cells, unipolar brush cells and numerous granule cells (Irie et al. 2006; Diño and Mugnaini 2008). Cartwheel cells (another major inhibitory interneuron) are located at the border of the molecular and pyramidal cell layers (Berrebi and Mugnaini 1991). The molecular or superficial layer contains a low density of small neurons (mainly composed of stellate cells) and neuronal processes including parallel fibres (unmyelinated granule cell axons), the spiny dendritic trees of cartwheel cells and the apical dendrites of pyramidal cells (Apostolides and Trussell 2014).

The development of the DCN has not been extensively studied. The few developmental studies have examined neurogenesis, neuronal migration and structural organization at a gross level in the mouse (Mlonyeni 1967; Webster and Webster 1980; Martin and Rickets 1981; Webster 1988; Ivanova and Yuasa 1998) while more detailed studies have mostly focused on the postnatal cat (Kane and Habib 1978), opossum

(Willard 1993) and other rodent species such as hamster (Schweitzer and Cant 1985; Schweitzer 1990; Schweitzer and Cecil 1992) and rat (Angulo et al. 1990). The popularity of using the mouse as an animal model for hearing studies has increased due to the availability of genetic mouse models of hearing disorders, such as the CD-1 mouse. The CD-1 mouse shows an early-onset progressive hearing loss that is initially associated with changes in the supporting tissues of the cochlea (Mahendrasingam et al. 2011). As part of an investigation into the development of the CN and effect of early peripheral degenerative changes on the CN, we report here the postnatal development of the CD-1 DCN at ages prior to major initial detectable peripheral degeneration at the gross level (Mahendrasingam et al. 2011). Here, we describe the overall morphological development, neuronal and neuroglial features and synaptic distribution using fluorescent Nissl staining and immunohistochemistry (IHC). We found that an adult-like structure of the DCN can be observed by the onset of hearing (P12).

MATERIALS AND METHODS

Animals and Tissue Preparation

CD-1 mice at eight postnatal time points (P0, P3, P6, P9, P12, P15, P18 and P21) with three to six animals at each time point were used for each experiment. Animals were euthanized with an intraperitoneal injection of sodium pentobarbitone (9 mg/mL; Nembutal, Virbac Laboratories, New Zealand) and transcardially perfused firstly with 0.9 % saline followed by 4 % paraformaldehyde (PFA; in 0.1 M phosphate buffer, PB; pH 7.4). The brains were removed and placed in the same fixative overnight at 4 °C after which they were cryoprotected in 30 % sucrose (in 0.1 M PB) at 4 °C for 2 days before being embedded in Optimal Cutting Temperature compound (Tissue-Tek® OCT; Sakura, USA) and cryosectioned in the parasagittal plane at 30 µm in a cryostat (Leica CM3050 S, Germany). Brainstem sections containing the DCN were collected in 0.1 M phosphate-buffered saline (PBS; pH 7.4) in 24-well plates for fluorescent Nissl staining and immunohistochemistry. All incubation steps were kept on a shaker to enable optimal labelling. All procedures were approved by the University of Auckland Animal Ethics Committee (AEC number R780).

Fluorescent Nissl Staining

Sections were washed in 0.1 % Triton®X-100 (in 0.1 M PBS) for 10 min to permeabilize the tissue for optimal staining. After washing in 0.1 M PBS (twice,

5 min each), the sections were incubated with diluted NeuroTrace™ 435/455 (1:10 in 0.1 M PBS; Invitrogen, USA) or NeuroTrace™ 500/525 (1:100; Invitrogen, USA) fluorescent Nissl stains for 30 min. Sections were then washed for 10 min in 0.1 % Triton®X-100 (in 0.1 M PBS) followed by washing in 0.1 M PBS (twice, 5 min each). Finally, the sections were washed in 0.1 M PBS for 2 h before mounting on glass slides with an anti-fading mounting medium (Citifluor, UK). All procedures were undertaken at room temperature.

Cell Dimension Measurement Cell dimensions were measured for readily identifiable cell types (namely pyramidal, cartwheel and granule cells) in the developing mouse DCN at each time point using the ruler tool in Adobe® Photoshop® CS5 (Adobe, USA) after calibration with the scale bar in each image. Because we were unable to track individual neurons across developmental ages to study the establishment of cell layers and neuronal types, the likely identity of cells was based on criteria that relied on soma shape/size and position within the DCN in Nissl-stained tissues (see below). Development-related changes in cell shape and nuclear chromatin staining patterns and positional changes associated with specific cell type migration paths limit the certainty with which the different cell types within the developing DCN can be identified. For example, our reliance on adult-type criteria excludes any immature cell that has yet to acquire the characteristics of the adult form. Because of these limitations, we restricted our analysis to those cell types for which robust Nissl-stain criteria were available for adult material (granule, pyramidal and cartwheel cells). Identification of pyramidal cells was further confounded by the fact that some pyramidal cells may have morphological similarities with giant cells of the DCN in histological sections (Zhang and Oertel 1994) and because of the expected difference in soma profile associated with differences in the level of sectioning and orientation of pyramidal cells. To avoid mislabelling giant cells as pyramidal cells, only those cells that contained a nucleus and had a fusiform-shaped soma with at least one of the two primary dendrites present in the sections were classified as pyramidal cells. We have referred to all the cell types in younger animals as “cell type-like” to reinforce the limitations in the interpretation of the qualitative and quantitative data on DCN neuronal development.

The long axes of the pyramidal cells were measured between the tapering points where the primary dendrites on either end were extending from the cell body and the short axes were taken at the widest point of the cell body perpendicular to the long axis. Cells exhibiting round to oval-shaped soma containing a relatively large nucleus often with multiple nucleoli

were classified as cartwheel cells (Hackney et al. 1990). Granule cells are the smallest cell type in the DCN and have a large nucleus that occupies almost the entire soma and exhibits a patchy chromatin pattern (Webster and Trune 1982). As cartwheel and granule cells are round to oval in shape, their diameters were measured along the longer axis. The number of animals used and the number of cells measured for each cell type are summarized in Table 1. Statistical analyses were performed using Prism 6 (GraphPad, USA) with one-way ANOVA and the Tukey-Kramer test for multiple comparisons of means. Values were indicated as mean±standard error of mean (mean±SEM). *P* values ≤0.05 were regarded as being significant.

Immunohistochemistry

Primary Antibodies Rabbit polyclonal anti-synaptophysin was purchased from Abcam, USA (ab68851; lot 862128), and used at 1:1000 dilution. Specific immunoreactivity of this product has been observed in several studies (Micheva et al. 2010; Aldahmash and Atteya 2011). Synaptophysin is a presynaptic vesicular glycoprotein that is abundant in the CNS (Bartolomé et al. 1993; Gil-Loyzaga et al. 1998).

Rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) was purchased from Abcam, USA (ab7260; lot GR104425-1), and used at 1:500 dilution. This antibody has been widely used in previous studies (e.g. Lee et al. 2011; Jansson et al. 2013). GFAP is an intermediate filament in most glial cells and has long been used as an astrocyte marker in the CNS (Eng 1985; Eng and Ghirnikar 1994; Eng et al. 2000). GFAP is expressed in several glial cell types including astrocytes, radial glia, ependymal cells and cerebellar Bergmann glial cells but has not been reported in microglia.

Mouse monoclonal anti-microtubule-associated protein (MAP2) was purchased from Millipore, USA (MAB364; lot NMM1581862), and used at 1:1000 dilution. This antibody has been used successfully to study dendritic morphology (Baxter et al. 2009; Jansson et al. 2013). MAP2 is a neuron-specific microtubule-associated protein that is enriched in the dendrites.

Procedures The following procedure applies to all antibodies except MAP2, which was raised in the same species as the experimental animal, and will be described separately. Each developmental series was processed together to ensure consistency. Sections were washed in 0.1 M PBS (twice, 5 min each), and non-specific immunoreactivity was blocked with 10 % normal horse serum (NHS) containing 1 % Triton®X-100 in 0.1 M PBS for 30 min at room temperature. This was followed by incubation in the

Table 1
Number of animals and cells used for measurements of dimensions in three DCN cell types

Age	Number of animals	Number of cells		
		Pyramidal cells	Cartwheel cells	Granule cells
P0	4	10	2	13
P3	5	12	9	13
P6	5	17	15	13
P9	5	19	9	13
P12	6	19	24	17
P15	3	14	16	16
P18	3	17	17	16
P21	6	13	26	21

primary antibody diluted in 5 % NHS containing 0.1 % Triton®X-100 in 0.1 M PBS at 4 °C for 36 h. Following primary antibody incubation, the sections were washed in 0.1 M PBS (3 times, 10 min each). Donkey anti-rabbit Alexa 594 secondary antibody (1:500; Invitrogen, USA) was applied in blocking solution (5 % NHS containing 0.1 % Triton®X-100 in 0.1 M PBS) for 1 to 2 h at room temperature. The sections were then washed in 0.1 M PBS (3 times, 10 min each) before mounting on glass slides with an anti-fade mounting medium (Citifluor, UK).

For MAP2 immunostaining, an additional blocking step was required to perform immunostaining with the mouse anti-MAP2 antibody on mouse tissue. Sections were collected in Tris-buffered saline (50 mM Tris-chloride and 150 mM sodium chloride; pH 7.6) containing 0.05 % Tween 20 (TBS-Tween 20) and washed in the same solution (twice, 2 min each). Non-specific antibody binding was blocked with 10 % normal goat serum (NGS) containing 1 % Triton®X-100 in 0.1 M PBS for 1 h followed by washing with TBS-Tween 20 (3 times, 2 min each). Endogenous mouse IgG was blocked with unconjugated AffiniPure Fab Fragment goat anti-mouse IgG (H+L) and unconjugated AffiniPure whole goat anti-mouse IgG (H+L) (#115-005-146, lot 99875 and #115-005-146, lot 98209, respectively, Jackson ImmunoResearch Labs, USA) at 1:50 dilutions for both in 0.1 M PBS, for 1 h at room temperature. The sections were washed with TBS-Tween 20 (3 times, 2 min each) before being incubated with the primary mouse anti-MAP2 antibody (in 5 % NGS containing 0.1 % Triton®X-100 in 0.1 M PBS) at 4 °C overnight. After washing the sections with TBS-Tween 20 (3 times, 2 min each), goat anti-mouse Alexa 488 secondary antibody (1:500; Invitrogen, USA) was applied and incubated for 1 h at room temperature. The sections were then washed with TBS-Tween 20 (3 times, 2 min each) and mounted on glass slides with anti-fade mounting medium (Citifluor, UK).

Double Labelling and Controls Sections labelled with anti-synaptophysin and anti-GFAP antibodies were

also labelled with fluorescent Nissl staining. Immunohistochemistry was performed followed by fluorescent Nissl staining (see procedures above). Some sections were also double labelled with anti-synaptophysin and anti-MAP2. In this case, because the primary antibodies of the two antibodies were raised in different species (rabbit and mouse, respectively), immunohistochemistry with the two antibodies was performed together as no cross-reactivity of the corresponding secondary antibodies was to be expected. The immunohistochemical procedures were identical to those described in [Procedures](#). No apparent difference was observed in double-labelled sections when compared to single-labelled sections with respect to each antibody; therefore, the specificity of the double labelling could be confirmed. For all immunohistochemistry experiments, a no-primary antibody control was included to verify the specificity of the secondary antibody.

Image Acquisition and Analysis

Sections were imaged by confocal laser scanning microscopy (Olympus FV1000 Live Cell System, Japan) using either a 20× (NA=0.75) or 60× (NA=1.35) objective. Most images presented are single optical sections. Occasionally, maximum intensity projection images were constructed for stacks of 10 (20×) or 20 (60×) optical sections (thicknesses of 1.14 or 0.46 µm, respectively). Confocal settings (including laser power, gain and offset) were all kept the same for each experimental run and for each antibody to ensure consistency. Images were enhanced uniformly per figure using Adobe® Photoshop® CS5 (Adobe, USA) for improved contrast.

Quantification of synaptophysin labelling was performed using ImageJ (National Institute of Health, USA). Low magnification (×20) maximum intensity projection images were used to outline the molecular layer and the deeper layers (pyramidal cell and deep layers combined) of the DCN and measure the mean intensity in each outlined area in five developmental

series. The difference in intensity was analysed across time using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. Two of the developmental series were used to take high magnification images ($\times 60$ with a 2 times digital zoom, final image size of 725×725 pixels) with a resolution of $0.146 \mu\text{m}/\text{pixel}$. Single optical sections at each age were colour thresholded using the "triangle" algorithm (ImageJ) to highlight the punctate labelling. The size of each puncta (measured in pixel^2) was measured using the "Analyze Particles" function, and then, the actual size of the puncta in μm^2 was calculated according to the size of the image (in number of pixels in the x and y -axes) and the actual size of the specimen in the image (in micrometres by micrometres). Three developmental time points (P3, P12 and P21) were chosen for quantitative analyses to span the important developmental period before and after the onset of hearing (P11/12).

RESULTS

Morphological and Neuronal Development of the DCN

Structural Organization and Cell Type Identification in the DCN of the Juvenile Mice. The organization of the CN at P21 is shown in Fig. 1. The DCN and VCN are separated by a layer of granule cells (gcl; Fig. 1A), and the molecular, pyramidal cell and deep layers of the DCN are quite distinctive at this age (Fig. 1B). Major cell types in the DCN were classified on the basis of their position, soma shape and size and nuclear chromatin staining pattern using fluorescent Nissl (see examples by arrowheads in Fig. 1B) using criteria described previously with classic Nissl stained

tissue (Webster and Trune 1982; Hackney et al. 1990) as described in the Materials and Methods section. Pyramidal cells were the easiest to identify due to their distribution in the DCN (i.e. forming a band in the pyramidal cell layer; Fig. 1B; red arrowheads). These cells often had fusiform-shaped cell bodies with two thick primary dendrites extending from opposite ends of the soma and commonly had one large, centrally located nucleolus. Giant cells were found in the deep layer and usually had large triangular-shaped cell bodies with three or more primary dendrites arranged in a radial pattern (Fig. 1B; yellow arrowhead). Cartwheel cells were classified based on their position along the boundary between the molecular and pyramidal cell layers and medium-sized round to oval-shaped cell bodies (Fig. 1B; green arrowheads). Cells classified as granule cells were the smallest cell type within the DCN, found most abundantly in the pyramidal cell layer. Their large nuclei typically contained several small darkly stained chromatin patches and almost completely occupied the cell volume thereby showing a characteristically reduced cytoplasm (Fig. 1B; the numerous small cells throughout the DCN but mostly in the pyramidal cell layer). The DCN also contains other cell types such as vertical cells, stellate cells, unipolar brush cells and Golgi cells, although they cannot be identified unequivocally using Nissl stain; so, they were excluded for this analysis.

Development of the Structural Organization and Major Cell Types in the Postnatal DCN. The predominant changes in histological organization occurred between P0 and P6 with little change thereafter (Fig. 2, left panel). At P0, the DCN was identifiable as a collection of cells of variable size underneath the floor of the fourth ventricle with no clear lamination (Fig. 2A1). By P3,

Fig. 1. Fluorescent Nissl staining of the brainstem in P21 mouse. **A** Parasagittal view of the brainstem revealing the spatial relationship of the major CN subdivisions. *Arrowhead* points to the ependymal cells overlying the DCN. **B** Parasagittal section of the DCN showing the three layers. *Arrowheads* point to examples several DCN cell types: pyramidal cells (*red*), cartwheel cells (*green*) and giant cells (*yellow*).

Both images are maximum intensity projection images. AVCN anterior ventral cochlear nucleus; Cb cerebellum; D dorsal; DCN dorsal cochlear nucleus; DL deep layer; gcl granule cell layer; mcp medial cerebellar peduncle; ML molecular layer; PL pyramidal cell layer; PVCN posterior ventral cochlear nucleus; R rostral. Scale bar in panel A = $100 \mu\text{m}$; in panel B = $20 \mu\text{m}$.

Fig. 2. Fluorescent Nissl staining of DCN and major cell types at selected developmental time points. *Left panel* overall morphological development of the DCN. **A1** P0. **B1** P6. **C1** P12. White dots indicate the boundary of the DCN at each age. Arrows point to the ependymal cells overlying the DCN. *Right panel* morphological appearance of major cell types (2, cartwheel cell; 3, granule cells; and 4, pyramidal cells) in the DCN at P0 (**A2-A4**), P6 (**B2-B4**) and P12 (**C2-C4**). All images are from single optical sections. Cb cerebellum; cp choroid plexus; DCN dorsal cochlear nucleus; DL deep layer; ML molecular layer; PL pyramidal cell layer; PVCN posterior ventral cochlear nucleus; vz ventricular zone. Scale bars in panels **A1**, **B1** and **C1**=100 μ m. Scale bars in panels **A2-A4**, **B2-B4** and **C2-C4**=10 μ m.

the first sign of the formation of the molecular layer was visible, and some large elongated cells similar in form to pyramidal cells could be identified (not shown). The three layers of the DCN emerged between P6 and P9: fusiform-shaped cells formed a more distinct layer by P6 while the molecular layer appeared as a narrow margin between the surface ependymal cells and the developing pyramidal cell layer and with relatively low cell density (Fig. 2B1). The DCN continued to expand in volume thereafter with no further apparent changes in the overall histological organization (P12; Fig. 2C1).

Although the DCN was poorly organized at P0, cells resembling the major cell types found in the adult

could already be recognized in fluorescent Nissl-stained sections. Examples of the cells that could be consistently classified into a cell type throughout the developmental time points (pyramidal-, cartwheel- and granule-like cells) are shown in Fig. 2 (right panel). Their cell dimensions were measured at each age examined (Fig. 3). Cartwheel-like cells with a spherical- to oval-shaped cell bodies and a nucleus containing multiple nucleoli were found as early as P0 (Fig. 2A2). Their size increased significantly with age (Fig. 2B2 and C2; $F_{(7,133)}=9.054$, $P<0.0001$, one-way ANOVA) with the most rapid growth occurring between P3 and P12 (Fig. 3; 24.6 % increase, $P<0.0001$, Tukey-Kramer test). Granule-like cells were classified as such by their small size, relatively large nuclei and granulated chromatin staining pattern (Fig. 2A3, B3 and C3). Their cell bodies did not change significantly in size during development (Fig. 3; $F_{(7,114)}=1.555$, $P=0.156$, one-way ANOVA), although they appeared to be less densely clustered at older ages (compare Fig. 2A3 and C3). Cells with fusiform-shaped cell bodies could be found at P0 and were classified as pyramidal-like cells. At this age, they were relatively small, and their nuclei usually contained more than one nucleolus (Fig. 2A4). Between P6 and P12, pyramidal-like cells had grown in size, and only one nucleolus was present in the nucleus (Fig. 2B4 and C4). Both the long and short axes of the pyramidal-like cells increased in length with age (long axis: $F_{(7,122)}=16.21$, $P<0.0001$; short axis: $F_{(7,122)}=8.732$, $P<0.0001$, one-way ANOVA) with the most rapid growth occurring between P0 and P9 (Fig. 3; 36.9 and 37.6 % increase for the long and short axes, respectively, $P<0.0001$, Tukey-Kramer test).

Development of Presynaptic Terminals

At P0 and P3, qualitative analysis of immunofluorescent images revealed diffuse synaptophysin labelling throughout the DCN with relatively low level of staining in the ventricular zone (Fig. 4A; P3). The overall level of labelling appeared to increase visibly during the first postnatal week, especially in the molecular layer, although it was still diffuse. Synaptophysin labelling was sustained throughout the DCN between P12 (Fig. 4B) and P21 (Fig. 4C). Quantification of synaptophysin labelling in the molecular and in the deeper layers (pyramidal cell and deep layers) at P3, P12 and P21 confirmed the qualitative observations (Fig. 4D1 and D2). In the molecular layer, the mean labelling intensity increased significantly ($P<0.05$) between P3 and P12 with no subsequent changes thereafter (Fig. 4D1). In contrast, the mean labelling intensity in the deeper

◀ **Fig. 3.** Measurements of cell dimensions of major cell types in the developing DCN. The dimensions of all cell types except granule cells increased with age ($P < 0.0001$, one-way ANOVA) (mean $\pm$ SEM, **** $P < 0.0001$).

layers did not significantly change from P3 to P12 or from P12 to P21 (Fig. 4D2).

The pattern of the synaptophysin labelling in the pyramidal cell layer was revealed when sections were double-labelled with fluorescent Nissl or anti-MAP2 (Fig. 5A to F). In young animals, small punctate synaptophysin labelling dominated and was mostly located in the neuropil (P3; Fig. 5A). Punctate synaptophysin labelling appeared on the surface of somata and proximal dendrites of predominately pyramidal-like cells by P9 (not shown) and the amount of labelling appeared to increase with age (Fig. 5E, P12; Fig. 5F, P21). By P12, labelling in the pyramidal cell layer revealed some clustering of synaptophysin terminals whose size appeared to have increased by P21 (Fig. 5B and C). These clusters appeared to be mainly associated with groups of granule cells. Quantification of the size of profiles labelled with synaptophysin was performed in two developmental series, and these data confirmed the qualitative observations. The relative (to P3) mean size of the profiles increased with age (Fig. 5G), and similarly, the relative fraction of the total area occupied by all labelled profiles, also increased over the developmental time period (Fig. 5H). The distribution of the size of the labelled profiles revealed a decrease in the number of small puncta between P3 and P12 (from ~ 4000 at P3 to ~ 2000 at P12 for puncta with area of less than $1 \mu\text{m}^2$; first bar in Fig. 5I). In addition, there was an age-related shift towards larger profiles although they were relatively fewer in numbers (Fig. 5I). These larger profiles correspond to the clusters of synaptophysin labelled profiles described above.

Development of Glial Cells in the Postnatal DCN

The development of glial cells in the mouse DCN was studied using an anti-GFAP antibody which labels astrocytes, radial glia and ependymal cells, among others (Eng et al. 2000) (Fig. 6). We observed that anti-GFAP labelling in newborn animals was mostly confined to the presumed ventricular zone (Fig. 6A). GFAP staining increased with age and was seen throughout the DCN at later stages (Fig. 6B, C and G).

GFAP staining revealed four histologically distinct profiles. At P0, long GFAP-positive processes oriented orthogonal to the floor of the fourth ventricle were occasionally observed within the DCN (Fig. 6A and D) but were no longer observed after this age. These long

Fig. 4. Development of synaptic distribution in the DCN. **A–C** Synaptophysin labelling of the DCN at P3 (**A**), P12 (**B**) and P21 (**C**). White dots in panel **A** outline the border of the tissue section surrounding the DCN. A no-primary antibody control is shown as an inset in panel **A**. All images are maximum projection images. **D1–D2** Measurements of synaptophysin labelling intensity in the

molecular layer (**ML**, **D1**) and in the deeper layers (**PL/DL**, **D2**) in five independent developmental series (* $P < 0.05$). **DL** deep layer; **ML** molecular layer; **PL** pyramidal cell layer; **PVCN** posterior ventral cochlear nucleus. Scale bar in panel **C** = 100 μ m and applies for panels **A** and **B**.

GFAP-positive processes are likely to originate from radial glia.

A second type of profile was observed at all ages examined and resembled tanycytes (a type of ependymal cell). They can be best characterized in material from P21 DCN. At this age, GFAP-positive labelling was seen surrounding the nucleus of cells of the ependyma (Fig. 6G, H and I). From there, processes extended towards the underlying DCN forming a network that sometimes reached the dorsal aspect of the pyramidal cell layer but did not penetrate deep into it. These structures could be detected as early as P0 but were only restricted to the ventricular zone (Fig. 6A). By P3, there was an apparent increase in the amount of GFAP staining in the ventricular zone (not shown). At P6, the GFAP processes began to invade the molecular layer, and these had reached the dorsal aspect of the pyramidal layer by P12,

with few changes thereafter. In a few cases, these processes were seen to be associated with blood capillaries (Fig. 6G, H and I; arrowheads) suggesting that at least some of these profiles could represent the presence of tanycytes.

Starting at P3, a different type of GFAP-positive fibre-like profile with a unique appearance was first seen in the DCN (see Fig. 6B and E). These GFAP-positive profiles had a tubular and hollow appearance with diameters of about 1 μ m (Fig. 6E and F). By P9 and P12, these GFAP-positive profiles had entered the deep and pyramidal cell layers, respectively (Fig. 6B and C), although they had a more disorganized spatial arrangement (Fig. 6F). Given their location and morphology, this type of GFAP-positive profile is likely to be associated with fibrous astrocytes.

Occasionally, cells consistent with the morphology of protoplasmic astrocytes (based on their star-like

Fig. 5. Development of synaptic distribution in the DCN pyramidal cell layer. **A–C** Anti-synaptophysin (red) double-labelled with fluorescent Nissl (blue) in the DCN pyramidal cell layer at P3 (**A**), P12 (**B**) and P21 (**C**). White circles indicate the clustered synaptophysin labelling. **E–H** Anti-synaptophysin (red) double-labelled with anti-MAP2 (green) in the DCN pyramidal cell layer at P3 (**D**), P12 (**E**) and P21 (**F**). White arrowheads in **F** outline punctate synaptophysin labelling around the pyramidal cell (Py). A no-primary antibody control for MAP2 labelling is shown as an inset in panel **D**. All images were single optical sections. **G–I** Quantification of synaptophysin labelling in the DCN pyramidal cell layer performed in two independent developmental series. All analyses were undertaken in 120× magnification images with a field of view of

106×106 μm², such as those shown in panels **A–C**. **G** Changes in the relative area of individual synaptophysin profile (“relative particle size”) with age with data normalized to P3 of the same developmental series. **H** Changes in the relative area of fraction occupied by all synaptophysin profiles in the region of interest with age normalized to P3 of the same developmental series. **I** Histograms from a representative developmental series showing the distribution of the size of synaptophysin profiles at P3 (*top panel*), P12 (*middle panel*) and P21 (*bottom panel*); y-axis break is between 15 and 1500; bin width=1 μm². Scale bar in panel **C**=20 μm and applies for panels **A** and **B**. Scale bar in panel **F**=20 μm and applies to panels **D** and **E**.

appearance) were observed to be in close contact with neurons in the molecular layer after P12 (Fig. 6G to I; arrows).

DISCUSSION

We have characterized the postnatal development of the CD-1 mouse DCN focussing on its overall histological organization, cellular and synaptic development between P0 and P21. We observed rapid maturation of most morphological features between P3 and the onset of hearing (P12) with little change subsequently (summarized in Fig. 7). This is, to our knowledge, the first detailed description of the postnatal morphological development of the mouse DCN at a cellular level.

Structural Organization and Neuronal Morphology Approach the Mature-Like State by the Onset of Hearing

The pattern of DCN morphological development in the CD-1 mouse is similar to that described in the hamster although key events are shifted by a few days, probably reflecting the later onset of hearing (P16) in the hamster (Schweitzer 1990; Schweitzer and Cecil 1992).

Qualitatively, we did not observe an increase in the size of the DCN until P3. Webster (1988) showed that the volume of the mouse DCN begins to increase sharply only after P6 and doubles in volume between P6 and P12. The volume increase coincides with reported increases in cell number (Mlonyeni 1967) which continue up to P12. Cell addition to the DCN is probably due to migration of already generated

Fig. 6. GFAP labelling in the developing DCN at selected time points. **A–C** Anti-GFAP labelled DCN at P0 (**A**), P9 (**B**) and P12 (**C**). White dots in panel **A** outline the border of the tissue section surrounding the DCN. A no-primary antibody control is shown as an inset in panel **C**. **D–F** Enlargements of the box in **A–C**, respectively. **D** A long GFAP-positive process in the DCN (arrowhead). **E** Parallel bundles of GFAP-positive processes ventral to the DCN deep layer. **F** GFAP-positive structures in the DCN pyramidal cell layer revealed tubular appearance (arrowhead). Arrow points to an example of a tubular GFAP structure in cross section. **G–I** Anti-GFAP labelling in the DCN at P21. “1” and “2” indicate two cells in the ependymal cell layer with cytoplasmic anti-GFAP labelling. Arrowhead points to a blood capillary. Arrow points to an astrocyte. **G** Anti-GFAP (red)/fluorescent Nissl (blue) double-labelled DCN molecular and pyramidal cell layers. **H** Enlargement of the box in **G** without fluorescent Nissl. **I** Enlargement of the bottom box from **G** with fluorescent Nissl. All images are maximum projection images except panels **H** and **I** which are single optical sections. 4th fourth ventricle; DL deep layer; ML molecular layer; PL pyramidal cell layer. Scale bar in panel **C**=100 μ m and applies to panels **A** and **B**. Scale bar in panel **D**=20 μ m, in panel **E**=100 μ m and in panel **F**=50 μ m. Scale bar in panel **G**=20 μ m, in panels **H** and **I**=10 μ m.

neuroblasts since all cell types of the DCN seem to be generated embryonically (Martin and Rickets 1981; Pierce 1967). Cell loss in the mouse DCN does not begin until around the onset of hearing (P12) at which time the rate of volume growth declines. Adult cell number is reached at around P16 (Mlonyeni 1967). The initial increase in DCN volume during development seems to reflect both the increase in cell number and cell size (this study and Mlonyeni 1967;

Webster 1988), whereas the slower increase in volume after P12 (Webster 1988) likely reflects the expansion of neuronal dendritic domains, genesis and growth of glial cells, increase in myelination and angiogenesis, especially considering the reduction of neurons due to naturally occurring cell death (Cant 1997).

On the basis of our data, morphological development of individual cell types in the DCN appears to occur at different rates. The onset of DCN neuronal growth coincides with ANF innervation (P0–P3; Schweitzer and Cant 1984; Mao 2013). The period of growth of other major principal cell types in the CN, which ends around P12 (Webster and Webster 1980 in Brugge 1983), has been suggested to be regulated by afferent activity (Pasic and Rubel 1989, 1991), and it is possible that, similarly, the ANFs may contribute to the initiation of cell growth of pyramidal and cartwheel cells in the DCN, particularly the pyramidal cells which receive direct ANF innervation. Although there is no evidence that cartwheel cells receive direct ANF innervation, they do respond to sound stimuli and electrical stimulation of the ANF nerve root albeit with longer latencies when compared to the principal cell types (Zhang and Oertel 1993; Parham and Kim 1995). Non-auditory inputs to cartwheel cells may also influence cartwheel cell size development, although our synaptophysin immunohistochemistry findings suggest that the mossy fibre inputs begin to mature in the DCN between P9 and P12. We cannot, however, rule out other as yet unidentified sources of input to cartwheel cells that are important for their development in the early postnatal days. Granule cells mainly receive mossy fibre inputs but not direct ANF innervation (Berglund and Brown 1994; Berglund et al. 1996; Benson and Brown 2004). In contrast to cartwheel and pyramidal cells, granule cell diameters did not change significantly with age. Further studies are needed to elucidate the role of ANFs in the development of cell size in the DCN.

Synaptic Inputs to the DCN May Be Reflected by the Developmental Changes in the Distribution of Presynaptic Terminals

In the newborn CD-1 mouse, synaptophysin labelling was dominated by punctate staining throughout the DCN. Although ANFs are already tonotopically organized within the immature VCN as early as E15.5 (Koundakjian et al. 2007), there is little evidence that ANFs have entered the DCN at this stage (Schweitzer and Cant 1984; Fariñas et al. 2001; Mao 2013). The punctate synaptophysin labelling, therefore, most likely represents diffuse presynaptic terminals between local connection neurons. Overall, the mean synaptophysin labelling intensity increased in the molecular layer before, but not after, the onset of

Fig. 7. Diagrams summarizing the structural development of the mouse DCN between P0 and P21. At P0, DCN cells form a cluster at the centre while the surface is covered by a thick portion of ependymal cells. Occasional GFAP-positive processes are seen in both regions running orthogonal to the DCN surface. Three DCN layers are apparent at P6. More GFAP-positive processes appear among the ependymal cells which occasionally extend into the molecular layer below. GFAP-positive processes with tubular structures also appear in the deep layer. By P12, the three DCN layers have expanded considerably and clustered synaptophysin profiles

start to appear around granule cells in the pyramidal cell layer. The GFAP-positive processes from the ependymal layer have extended deeper into underlying molecular layer, some of which are seen to contact blood vessels, while the tubular processes have extended into the pyramidal cell layer. At P21, the DCN has grown further and clustered synaptophysin profiles around granule cells have increased in size. GFAP-positive processes from the ependymal cell layer has reached the upper portion of the pyramidal cell layer while the tubular processes have become more extensive within the pyramidal cell layer.

hearing. This increase may reflect the maturation of the local circuitry in this region, which involves inputs from parallel fibres and local interneurons such as stellate and cartwheel cells (Berrebi and Mugnaini 1991; Mugnaini et al. 1980b). In contrast, there was no measurable change in mean synaptophysin labelling intensity in deeper DCN layers (pyramidal cell and deep layers). It must be noted, however, that since the DCN volume increases during this time, a lack of change in synaptophysin labeling intensity should not be interpreted as an arrest of synaptogenesis in the DCN deeper layers. There was an increase in average synaptophysin labelled particle size during the first two postnatal weeks, although the fractional area occupied by all synaptophysin profiles did not appear to increase until after the onset of hearing. Clustered synaptophysin labelling associated with granule cells and resembling the glomerular synaptic terminals typical of mossy fibres (Mugnaini et al. 1980b; Zhou and Shore 2004; Haenggeli et al. 2005) were seen by P12, and their size and abundance increased over the following week. Taken together, the results of our synaptophysin labeling suggest that there is significant synaptic reorganization in the DCN of CD-1 mice following the onset of hearing and coinciding with the period of neuronal cell death (Mlonyeni 1967). More details regarding the origin of these synaptic inputs are needed to help interpret the significance of these observations. For example, it is not known when the mossy fibre terminals arrive in the DCN. It is possible that they arrive early and gradually develop into the glomerular morphology during the first two postnatal weeks, as in the case of the endbulb of Held in the

VCN (Limb and Ryugo 2000). Future studies exploiting that the ANFs and spinal trigeminal inputs utilize different vesicular glutamate transporters (VGLUT1 and VGLUT2, respectively) (Zhou et al. 2007) may provide additional information for the developmental timelines of the DCN synapses of ANF or somatosensory origin.

Several Glial Cell Types Are Differentially Expressed in the DCN During Development

The morphology and distribution of GFAP-positive structures in the DCN were both region- and age-specific suggesting multiple glial cell types. The few long GFAP-positive processes observed at P0 which were oriented orthogonal to the DCN surface most likely belong to radial glia that are known to assist in the neuronal migration of large multipolar neurons in the DCN during embryonic development (Ivanova and Yuasa 1998). The long processes observed in this study were not seen during the time when neuronal numbers are reported to increase in the DCN (up to P12; Mlonyeni 1967), suggesting that later migrating cells do not rely on radial glia. Thus, neuroblasts that populate the DCN may rely on different migratory mechanisms.

GFAP staining was also observed in the ventricular zone/ependyma where it surrounded the nuclei of some ependymal cells and labelled processes extending into the DCN. In older animals, some of these processes were seen to contact blood capillaries and neurons, suggesting the possible presence of tanycytes in the DCN. Tanycytes are possibly involved in

communication between the cerebrospinal fluid, neurons and blood vessels, and whilst abundant in some other parts of the brain (Felten et al. 1981), they have not been reported in the DCN, and this warrants further study.

In regions ventral to the molecular layer, GFAP-positive processes with a hollow appearance were seen, and their diameter suggests that they may represent structures, such as fibrous astrocytes, wrapping axons (Shehab et al. 1990). In support of this, the internal diameters of these tubular processes match those of typical axons (~1 μm). The appearance of these hollow structures coincides with the timing of spiral ganglion neuron myelination (Schweitzer and Cant 1984; Romand and Romand 1985), suggesting possible association between oligodendrocytes and astrocytes which have been shown to interact via gap junctions for metabolic support (Nagy and Rash 2000).

The occasional star-shaped GFAP-positive cell observed in the molecular layer resembled protoplasmic astrocytes, which normally reside in the gray matter and have richly arborized processes with end-feet enveloping synapses (Shehab et al. 1990). Most glial cells are generated perinatally in the mouse (Martin and Rickets 1981).

Here, we indicate the progressive appearance of GFAP-positive processes from P0 to P21, starting from the ventricular zone/ependyma to the deep layer followed by the pyramidal cell layer. The deep layer to pyramidal cell layer developmental pattern of the tubular processes may relate to the ingrowth of the ANFs into the DCN and/or their myelination. It is important to note that as GFAP does not label all types of glial cells (Malhotra et al. 1989), it is likely that the findings in the current study only represent the maturation of a subset of glia and the glial cell maturation and development in the DCN needs to be studied further.

SUMMARY

This study provides the first detailed description of the morphological development of the mouse dorsal cochlear nucleus and shows that the most rapid changes in structural organization, cellular morphology and synaptic distribution occur during the first two postnatal weeks and slows down considerably thereafter (Fig. 7). The relatively rapid development of the DCN precedes the “onset of hearing” in the mouse (P11–12) (Song et al. 2006; Manderson 2010). It has been proposed that the development of afferent synaptic connections in the central auditory system occurring before the onset of hearing is driven by

spontaneous activity in the immature auditory periphery (Tritsch et al. 2007; Tritsch and Bergles 2010), and this may influence the early developmental changes in the DCN observed in the current study before the onset of hearing. The later development of the mossy fibre terminals suggests that acoustic input may be important for the maturation of the non-auditory/descending inputs. Thus, different aspects of the development of the DCN may depend on different modes of afferent regulation.

Hearing in the CD-1 mouse begins to decline about 3 weeks after birth; thus, the observation that the DCN has a normal histological appearance at least up to P21 suggests that the hearing phenotype of the CD-1 mouse does not appear to manifest itself as early central abnormalities. With the developmental sequence now defined in this strain, we intend to investigate the impact of peripheral changes and early onset hearing loss on the neural circuitry in the CN.

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Conflict of Interest

The authors declare no conflict of interest.

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