Original article

Morphogenesis and maturation of synapses in developing human cochlear ganglion

Sethi, M.; Mishra, S.*; Kaul, JM. and Vasudeva, N.

Maulana Azad Medical College (Anatomy), 110002, New Delhi, Índia
*E-mail: sabitamishra12@gmail.com

Abstract

A light microscopic study of the cochlear ganglion development at various stages of gestation was conducted in human fetuses to understand its morphological maturation with particular emphasis on synaptogenesis. Knowledge of development of the peripheral auditory system especially the cochlea and the cochlear ganglion contributes to understanding the effects of ototoxic insults on hearing function. Ten aborted fetuses from 14th to 28th weeks of gestation were procured from Department of Obstetrics and Gynaecology, LN Hospital after obtaining ethical clearance and were analyzed under light microscopy. Evidence of synaptogenesis was demonstrated by using an Immunohistochemical stain for Synaptophysin, a 38kD integral membrane protein of synaptic vesicles. A progressive increase in neuronal size was revealed from 16th to 28th weeks of gestation. At 20th weeks gestation, neuronal processes and the soma were surrounded by supporting cells giving a honeycombed pattern. Myelinated neuronal processes were seen using Luxol fast Blue stain by 24wks of gestation, however, the perikarya showed surrounding myelination at 28th weeks in some of the neurons. The expression of Synaptophysin in cytoplasm and nerve fibers grew stronger gradually from 20th weeks till 28th weeks of gestation. Thus maturation of human cochlear ganglion commences from mid gestation and almost an adult pattern was seen at 28th weeks.

Keywords: cochlear ganglion, morphogenesis, synaptogenesis.

1 Introduction

The incidence of congenital deafness has increased appreciably in developing countries as a result the requirement of hearing aids has gone up to 32 million in a year (KUMAR, 2000). About one in thousand children each year are born with an auditory deficiency which can be cured by means of a cochlear implant. Prior to the cochlear implantation, the surgeon evaluates the developmental status of the inner ear to diagnose rare cochlear or vestibular anomalies like cochlear nerve aplasia, cochlear agenesis, which can preclude implantation (FISHMAN and HOLLIDAY, 2006).

The auditory pathway has a hierarchical arrangement of nuclei and tracts. Auditory stimuli are received by the hair cells of the organ of Corti situated in the membranous cochlea; these act as mechano receptors. Cell bodies of the processes making contact with the peripheral inner and outer hair cells are clustered in the cochlear ganglion. The bony channel that contains these cell bodies travels in a spiral direction towards the apex of the cochlea and is called Rosenthal's canal. Within the cochlear ganglion are present - myelinated nerve fibers which originate from afferent bipolar cell bodies, and en passant efferent nerve fibers contained in a discrete bundle called the intraganglionic spiral bundle. Central processes of the ganglion cells forms the cochlear nerve and relay in the cochlear nucleus in the brainstem. The next sets of fibers relay in some of the nuclei along the pathway before reaching the inferior colliculi and medial geniculate body. Final destination for auditory reception is the auditory cortex in the temporal lobe (KANDLER and FRIAUF, 1993).

The cochlear ganglion neurons thus transmit acoustic information between inner ear and the brain. The cochlear ganglion consists of neuron cell bodies of two types- Type I and Type II, well characterized by their structural, ultrastructural and biochemical features and their connection with receptors (CAROL and KIMURA, 1980). Ultrastructural study of ganglion cell neurons demonstrate that approximately 94% of neurons are Type I and remaining 6% are type II. The type I neurons are large bipolar with a large spherical nucleus, the cytoplasm has abundant mitochondria, numerous endoplasmic reticulum, ribosomes and nissl bodies and is myelinated. On the other hand the type II ganglion neurons are small bipolar or pseudounipolar neurons. They have a small eccentric lobulated nucleus, cytoplasm is filamentous due to the presence of neurofilament lying loose and in discrete bundles. They have either a thin myelin sheath or are unmyelinated. Around ten type I cochlear ganglion neurons innervate one inner hair cells, while one type II ganglion neuron innervates ten outer hair cells. The neurons of the cochlear ganglion are derived from the otic placode and produce a topographically precise pattern of connection throughout the auditory pathway. (GRAVEN and BROWNE, 2008).

In humans, the cochlea starts functioning in prenatal period at around 22 weeks of gestation (PUJOL, LAVIGNE-REBILLARD and UZIEL, 1990, 1991) and auditory brainstem evoked potentials have been elicited between 19-28 weeks of gestation. (LARY, BRIASSOULIS, DEVRIES et al., 1985; HEPPER and SHAHIDULLAH, 1994). Neurosensory part of the auditory system develops primarily after 20 weeks of gestational age in human (GRAVEN and BROWNE, 2008). The auditory system

becomes functional when the processes of the cochlear ganglion neurons connect inner hair cells to the brain stem and finally the auditory cortex (HALL III, 2000). Hence development of cochlear ganglion neurons is one of the most significant steps in connecting the developing peripheral auditory apparatus with the central auditory circuitry.

According to reports lower animals like rats, mice gerbils are born deaf, on the contrary human fetuses are able to hear prenatally (DWORNICKA, JASIENSKA, SMOLARZ et al., 1964; BARDEN, PELTZMAN and GRAHAM et al., 1968; SAKABE and ARAYAMA, 1969; GRIMWADE, WALKER, BARTLETT et al., 1971; GOODLIN and SCHMIDT, 1972). Morphogenesis and maturation of innervation patterns in developing cochlear ganglion are well documented in lower animals (SCHWARTZ, PARAKKAL and GULLEY, 1983; ROMAND and ROMAND, 1984; HAFIDI and ROMAND, 1989; HAFIDI, DESPRES and ROMAND, 1990; ROMAND and ROMAND, 1990; ROMAND, SOBKOWICZ and EMMERLING, 1990; BERGLUND and RYUGO, 1991; HAFIDI, DESPRES and ROMAND, 1993; WHITEHEAD and MOREST, 1985; SOKOLOWSKI and CUNNINGHAM, 1999; HE and YANG, 2011), while human studies are scanty. Hence the present study was undertaken to understand significant aspects of prenatal development of human cochlear ganglion. Morphological maturation and, myelination were observed by light microscopy. Synaptogenesis was observed by using a monoclonal antibody against Synaptophysin, a 38kDa membrane bound protein found in synaptic vesicles.

2 Materials and Methods

2.1 Fetus collection

Ten aborted human fetuses aged between 14-28 weeks of gestation were procured from the Department of Obstetrics & Gynaecology, Lok Nayak Hospital, New Delhi, India after obtaining approval from the Institutional Ethical Committee.

The fetuses less than 20 weeks gestation (WG) were obtained from cases where Medical termination of pregnancy was conducted under the MTP Act, while those more than 20WG were cases of spontaneous abortions and still births. None of the mother suffered from any medical illness during pregnancy and the fetuses used in the study had no congenital anomalies.

Each fetus was immediately preserved within 4 hours of delivery to minimize postmortem changes. The fetuses were weighed and important parameters like the Crown rump Length, Crown Heel length, Foot length and Biparietal Diameter were measured to determine the foetal age (SAILAJA, AHUJA and GOPINATH,1996).

2.2 Tissue preparation

After fixation brain was removed and petrous part of temporal bone was dissected out. Specimens from higher gestational ages were decalcified in EDTA. Specimens were labeled and processed for paraffin embedding. Seven micron thick serial sections were generated on a rotary microtome with the anterior surface of the petrous temporal bone as the cutting surface along its superior border.

2.3 Staining

Sections were stained with hematoxylin and eosin for determination of morphological landmarks. Nissl's stain was used for morphology and maturation of neurons. Luxol fast blue stain with neutral red as counterstain was used to observe myelination.

2.4 Immunohistochemistry

The sections after being rinsed with Phosphate Buffer Saline (PBS) were incubated in PBS with 0.1% Triton X for 40 min at room temperature and then with a blocking solution containing 10% normal horse serum for 30 min at room temperature. Sections were then incubated overnight with a monoclonal primary antibody against Synaptophysin in 1:200 dilution in PBS at 4 °C. The antigen-antibody reaction was observed using secondary antibody tagged with DAB as the chromogen.

All sections were examined under a BX61 computerised microscope and images were captured by DP71 camera and analysed by image Proplus soft ware.

3 Results

In 14weeks embryo the ganglion was seen as a single circumscribed collection of neurons in relation to the mesenchymatous cochlear duct (Figure 1a). In the ganglion a cluster of neurons and supporting cells were observed (Figure 1b). The neurons were small, immature and hardly displayed any cytoplasm. They had a euchromatic nucleus with prominent nucleolus. Large number of neuroglia having darkly stained spindle shaped nuclei were also observed. (Figure 1c).

Cell processes at this gestational age was not evident by luxol fast blue stain, the nucleus of neurons and neuroglia stained red by the counter stain. (Figure 1d)

At 16 weeks of gestation scala vestibuli and scala tympani of cochlear duct were clearly defined (Figure 2a). The cochlea was cartilaginous with the ganglion close to it. The ganglion neurons formed groups with intervening nerve fibres. The neurons were scattered with no uniform distribution Large number of neuroglial cells were also observed. (Figure 2b). In Nissl's stain the neurons had a thin rim of cytoplasm. All the neurons were small in size. Most of the neurons showed thin processes. Synaptophysin immunoreactivity was not observed at this age.

In the 20 weeks embryo the ganglion increased in size and the cochlear duct had taken its final shape with the appearance of ossification (Figures 3a, b).The neurons were arranged in a clusters and were of varied size; with small and large sized neurons uniformly present. In Nissl's stain neurons showed developing processes and ganglion showed a typical honeycombed appearance (Figure 3c). Luxol fast blue stain confirmed the evidence of myelination by staining of the nerve fibres. Neurons and supporting cells stained pink with the counterstain and they were smaller in size (Figure 3d). The expression of synaptophysin was very weak (Figure 3e).

By 24 weeks of gestation multiple cut sections of the cochlear duct were seen with a very well developed ganglion (Figure 4a). The cartilage is ossified to form the bony cochlea. The ganglion was located adjacent to each cut section of cochlear duct with peripheral processes towards

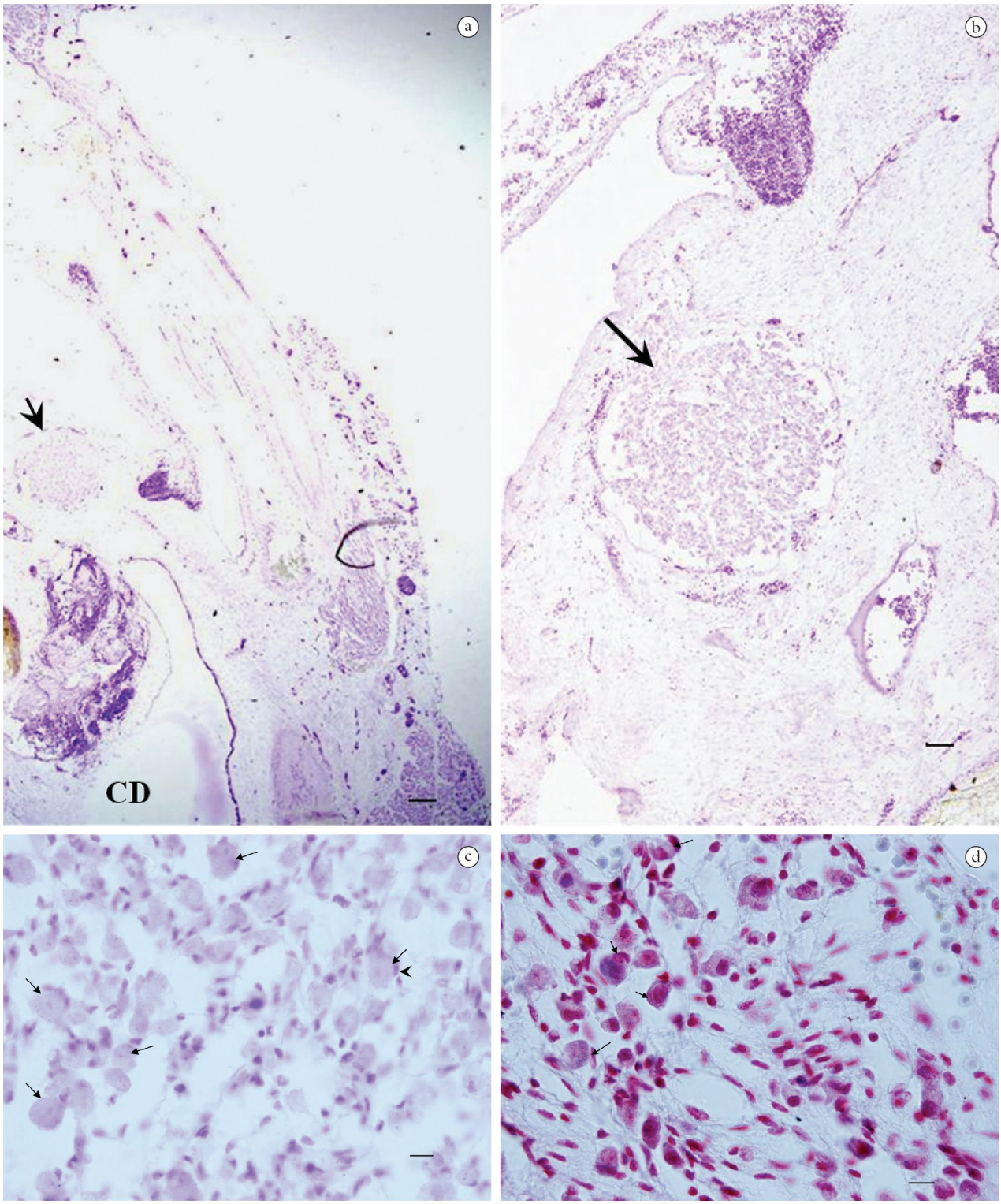

Figure 1. a) Photomicrograph of mesenchymatous cochlear duct (CD) with the cochlear ganglion (G). Age-14weeks. (Nissl's stain) Scalebar: 500 µm. b) Photomicrograph of cochlear ganglion seen as single circumscribed collection. Age-14weeks, (Nissl's stain) Scalebar: 50 µm. c) Photomicrograph showing the neurons having large nucleus, prominent nucleolus(arrow) and scanty cytoplasm. Neurons are surrounded by supporting cells (arrowheads). Age-14weeks, (Nissl's stain) Scalebar: 10 µm. d) Photomicrograph showing neurons stained pink, no processes or fibers can be seen at this stage. Satellite cells (arrows) surrounding the neurons. Age-14weeks, (Luxol Fast Blue stain) Scalebar:10 µm.

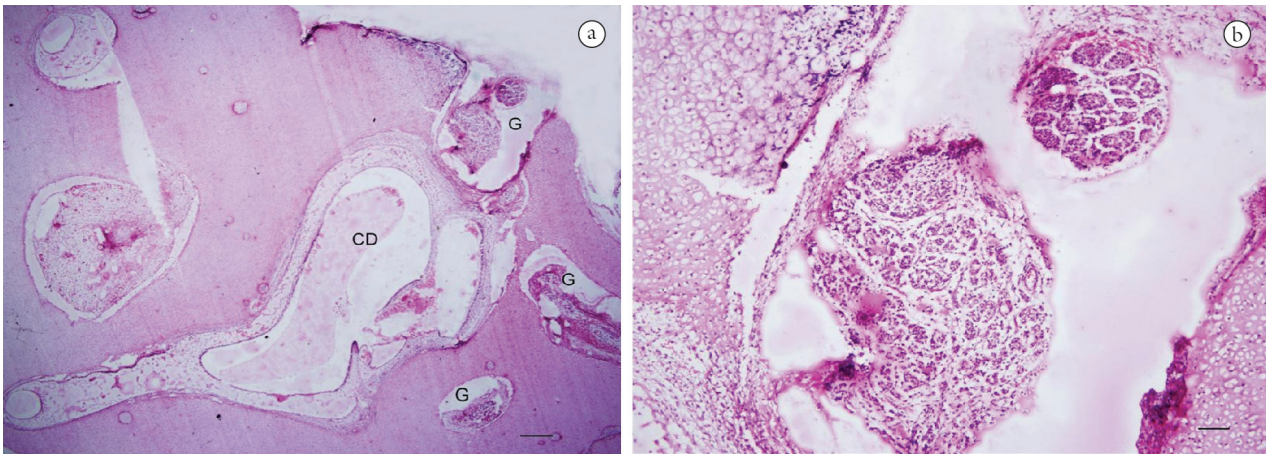

Figure 2. a) Photomicrograph showing the ganglion (G) seen as circular circumscribed collections of neurons around the cartilaginous cochlear duct (CD). Age- 16weeks, (H&E staining) Scalebar:1000 µm. b) Photomicrograph with the scattered ganglion neurons without any uniform distribution. Age- 16weeks, (H&E staining) Scalebar: 100 µm.

the organ of Corti and the central processes forming a bundle of cochlear nerve. Neurons appeared large and bipolar with fully developed processes and coarse nissl's granules with cresyl violet staining (Figure 4b).

Myelination was observed at this gestational age as most of the fibres were stained blue by luxol fast blue stain. Pink stained supporting cells were seen surrounding each nerve fibre (Figure 4c). The fibres were scattered and not arranged as discrete bundles. A few of the neurons showed a bluish hue around the perikarya and were surrounded by a layer of pink stained supporting cells. The expression of Synaptophysin was enhanced as compared with the lower age group (Figure 4d).

By 28 weeks of gestation the ganglion was sufficiently mature having large size neurons and intervening nerve fibres with supporting cells (Figures 5a, b). Cytoplasm showed fine Nissl's granules depicting sufficient achievement of neuronal maturation (Figure 5c).

Nerve fibres were stained blue with luxol fast stain showing myelination and were surrounded by supporting cells. These fibres were seen coursing in between the neurons in bundles (Figure 5d). A few of the neurons were seen surrounded by bluish hue and an oval pink stained nucleus of supporting cell seen as a cap around it (Figure 5e).

The Synaptophysin immunoreactivity was intense at 28th weeks of gestation. Nerve fibres showed beaded appearance and the neurons showed perineuronal labeling with synaptophysin (Figures 5f, g).

4 Discussion

This study has emphasized on the morphological maturation of cochlear ganglion and indirectly assesses the functional maturation of synapses in the human fetus.

To the best of our knowledge this is one of the first reports where along with light microscopic observations; synaptophysin expression has also been observed in the developing human cochlear ganglion.

According to literature, the first sign of differentiation of the vestibular and cochlear ganglion has been observed as early as 4th week (WOZNIAK, BRUSKA, ULTAOWSKA-BLASZYK et al., 1993). In the present study the lowest gestational age fetus was of 14th week, where the cochlear ganglion had already separated from the vestibular ganglion and was apparent by this age.

4.1 Morphological maturation of ganglion

The ganglion neurons have a double source of origin, both from the otocyst and the neural crest evidenced by the otocystic and neural crest theories (YNTEMA, 1937; D'AMICO-MARTEL and NODEN, 1983). The ganglion seen in approximation to the cochlear duct has supported the otocystic theory and the presence of Schwannoblast cells along the nerve processes and in the matrix has supported the neural crest theory as mentioned by Sánchez del Rey, Sánchez Fernández, Martínez Ibarguen et al. (1995). They also suggested that interaction between modiolar ossification and organizing nerve processes resulted in increase in distance between the ganglion and the cochlear duct in late fetal stages. Cochlea is the first site where ossification begins in the petrous part of temporal bone (NEMZEK, BRODIE, CHONG et al., 1996). Between 18-24weeks of gestation there is rapid apposition of shell of ossification around cochlea, which is the time period by which the ganglion is morphologically mature.

In accordance with the previous literatures cited, by 14th week of gestation the ganglion neurons were disorganized (SÁNCHEZ DEL REY, SÁNCHEZ FERNÁNDEZ, MARTÍNEZ IBARGUEN et al., 1995). Our results reaffirmed these finding as the neurons by 14th weeks of gestation didn't show any uniform distribution. The population of large ganglion neurons increased gradually in subsequent week fetuses. The fetus of highest gestation age in our study was of 28 week gestation, in which the population of large ganglion neuron was more than the small neurons. Increase in the size of the neurons also emphasized that the cytoplasmic nuclear ratio increases with advance in gestational age. It was not possible to differentiate the type I and type II ganglion neurons by light microscopy, as one would require ultrastructural and specific neurofilament marker for identification. Infact type I and Type II neurons can only be distinguished late in

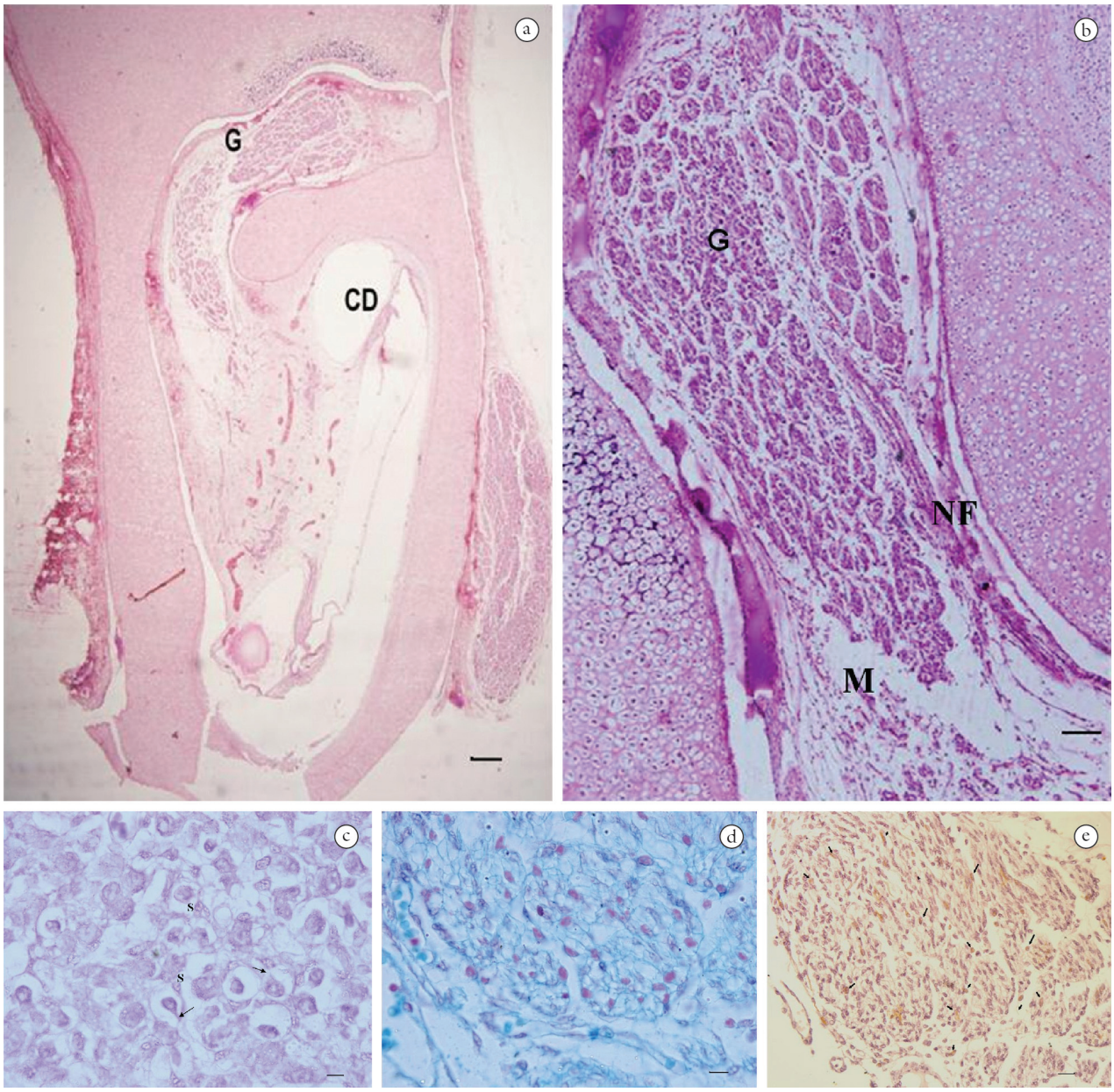

Figure 3. a) Photomicrograph showing the ganglion (G) adjacent to the developing cochlear duct(CD). Age-20weeks, (H&E stain) Scalebar: 500 µm. b) Photomicrograph with the ganglion (G) present in the spiral modiolus(M). Nerve fibers (NF) are seen emerging from within the ganglion. Age-20weeks, (H&E stain) Scalebar: 50 µm. c) Photomicrograph showing coarse Nissl's substance in the neuronal cytoplasm. Cell processes (arrows) are evident. Supporting cells (S) are surrounding the neurons. The ganglion is giving a typical honeycombed appearance. Age- 20weeks, (Nissl's stain) Scalebar:10 µm. d) Photomicrograph with the neurons stained pink. Processes and nerve fibers are stained blue. Age-20weeks, (Luxol fast blue stain) Scalebar: 25 µm. e) Photomicrograph showing faint expression (arrows) of synaptophysin in some of the neurons. Scalebar: 25 µm.

development when the course of their peripheral dendrites can be tracked to the different types of hair cells (PERKINS and MOREST, 1975; SIMMONS, MANSON-GIESEKE, HENDRIX et al., 1991) or when the first sign of myelination can be seen on Type I cells (PUJOL and HILDING, 1973; ROMAND, ROMAND, MULLE et al., 1980; ROMAND and ROMAND, 1982; SCHWARTZ, PARAKKAL and GULLEY,1983). Even immunocytochemistry, which in adulthood helps to differentiate the two types of neurons, is not particularly useful at the early stages. In the cochlea of human neonates the proportion of Type II vs Type I neurons is significantly greater than in adults (CHIONG, BURGESS and NADOL, 1993). Studies in mouse have demonstrated that there is no significant postnatal change in Type I neuron density while there occurs selective loss of Type II ganglion neurons with their proportion decreasing from 25% in early embryogenesis to 12% in infant cochlea (BARCLAY, RYAN and HOUSLEY, 2011).

At 16th week of gestation and thereafter, the number of neurons demonstrated a progressive increase in size with

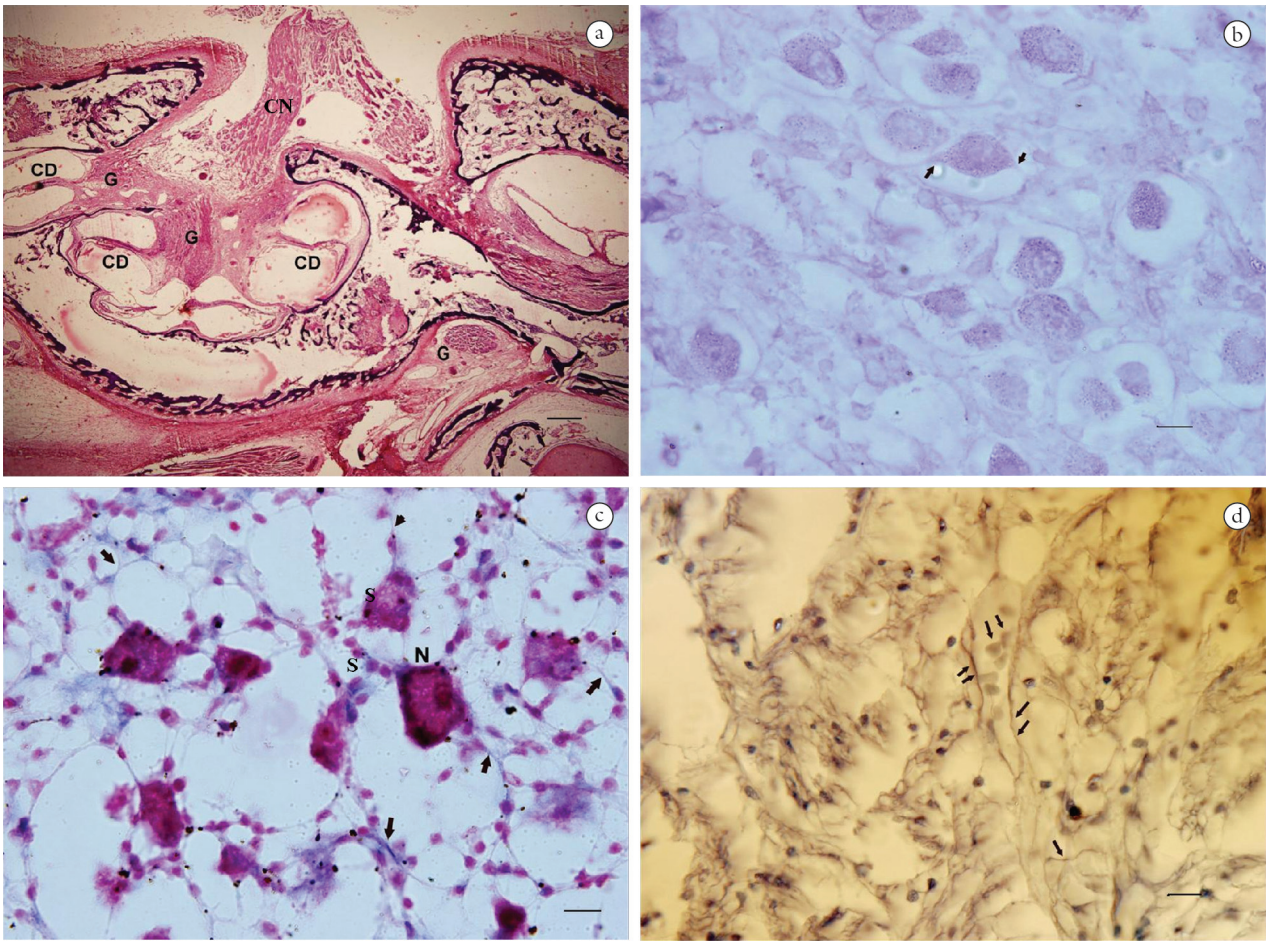

Figure 4. a) Photomicrograph showing cut sections of various turns of cochlear duct (CD) seen along with the ganglion (G) lying in its close proximity. Bundle of cochlear nerve (CN) is seen. Age-24weeks, (H&E stain) Scalebar:1000 µm. b) Photomicrograph showing bipolar neurons with its processes (arrows). All the neurons have well formed Nissl's substance in their cytoplasm. Age-24weeks, (Nissl's stain) Scalebar:10 µm. c) Photomicrograph with the neurons (N) stained dark pink and having a bluish periphery. Fibers and Processes (arrows) are stained blue. Supporting cells (S) seen as small round pink stained cells surrounding neurons and lying along the fibers. Age-24weeks, (Luxol fast blue stain)) Scalebar:10 µm. d) Photomicrograph showing expression of synaptophysin in nerve fibers (arrows) giving them a beaded appearance. Scalebar: 25 µm.

visible cytoplasm and appearance of Nissl's substance. Mature neurons with coarse Nissl's granules were seen at 20 weeks of gestation and presence of fine Nissl's substance was seen at 24 weeks of gestation. Previous reports shows presence of structurally mature cochlear ganglion by 20 weeks of gestation (BIBAS, HORNIGOLD, LIANG et al., 2006). In sympathetic ganglia also Nissl's substance was identified at 16th week of gestation (KIRAN, 2002). This is in confirmation to the present study. Thus it can be said that cytoplasmic maturation was complete by 28th week gestation. Appearance of Nissl's substance could be explained due to an increase in metabolic activity.

Cell processes started appearing by 16th weeks of gestation, more prominent processes surrounded by supporting cells were seen with subsequent maturation of the ganglion. Studies have showed that processes surrounded by supporting cells appeared at 15 weeks of gestation (BIBAS, HORNIGOLD, LIANG et al., 2006). The nerve fibers which were coursing through the ganglion were forming groups. These fibers were more distinct by 20th week of gestation. At 24th week of gestation fibers were very well evident as bundles and these could be the intra ganglionic spiral bundles. Light microscopy studies have revealed appearance of nerve fascicles by 12 weeks of gestation, initially arranged loosely but later compacted by 18 weeks of gestation. The association of axons with darkly stained supporting cells was also observed at 18weeks gestation (RAY, ROY, WADHWA et al., 2005).

4.2 Myelination

Morphological techniques for assessing the onset of myelination are an index of maturity in the auditory pathway. The entire process of myelination is sequential (MOORE, PERAZZO and BRAUN, 1995).

In lower animals majority of cochlear ganglion neurons were myelinated. Myelination in mouse begins in peripheral and central fibers, and finishes in the spiral ganglion neuronal soma during postnatal days 3 to 5 in a basal to apical direction (ANNIKO, 1983). Evidence of first myelin sheaths were detected in kittens between 4th and 6th postnatal day, around

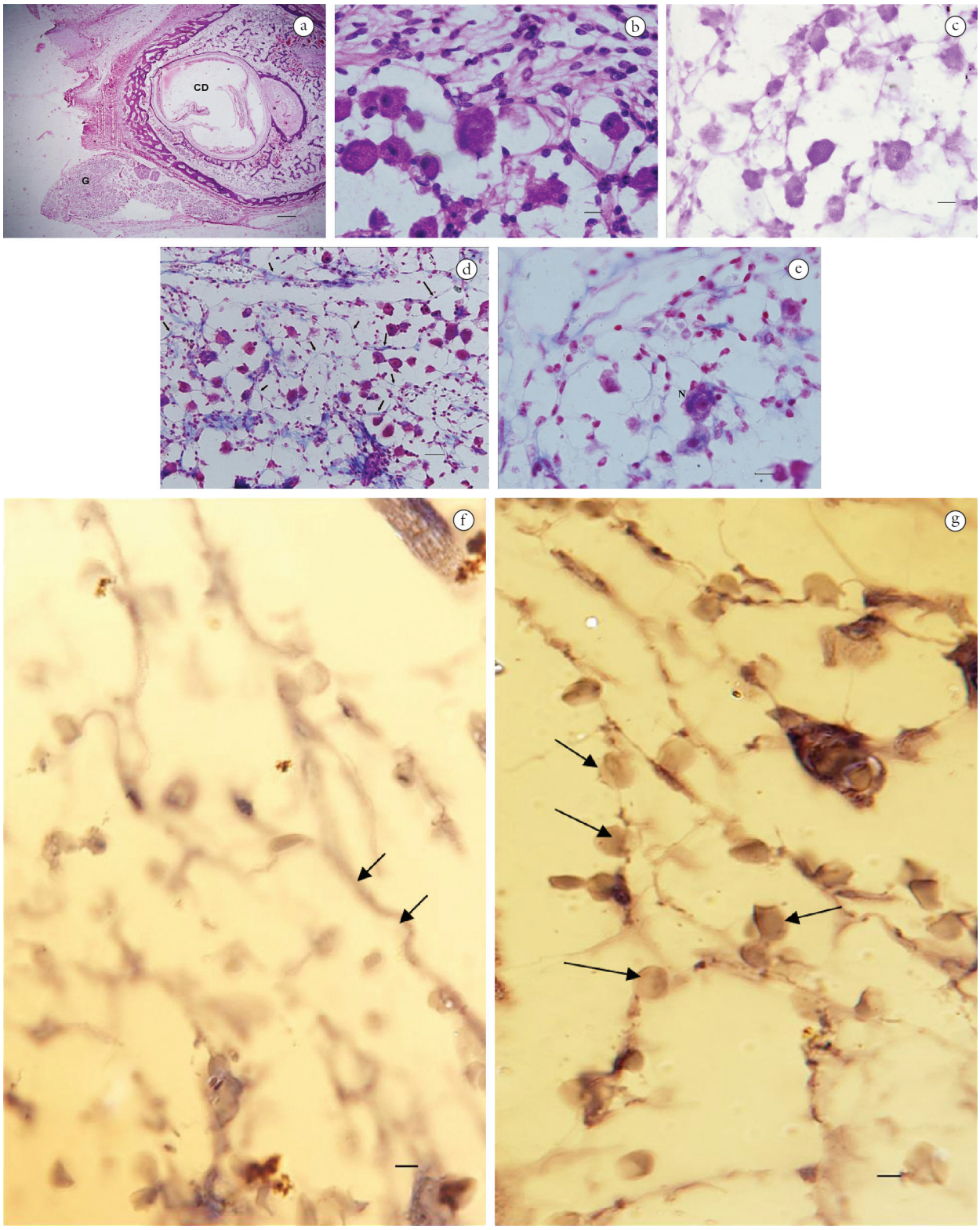

Figure 5. a) Photomicrograph showing well formed cochlear duct (CD) with the adjacent ganglion (G) (H&E stain)). Scalebar: 1000 µm. b) Photomicrograph with large sized neurons having abundant cytoplasm. Supporting cells(S) seen as a single sheet around the neuronal soma and also surround the nerve fibers. (H&E stain)) Scalebar:10 µm. c) Photomicrograph showing large mature neurons having large nucleus, abundant cytoplasm and fine granular Nissl's substance in the cytoplasm (Nissl's stain)) Scalebar:10 µm. d) Photomicrograph of the neurons stained dark pink and the supporting cells stained light pink. Nerve fibers (arrows) are stained blue (Luxol fast blue stain)) Scalebar: 25 µm. e) Photomicrograph of few Neuron (N) having a bluish periphery suggesting evidence of myelination) Scalebar:10 µm. f) Photomicrograph with enhanced expression of synaptophysin in the nerve fibers (arrow) giving them a varicose appearance) Scalebar:12 µm. g) Photomicrograph with the neurons having dark brown perineuronal expression of synaptophysin at their periphery (arrows). Scalebar:12 µm.

peripheral and central fibers and later on 6th-8th postnatal day around the neuronal soma (ROMAND, SOBKOWICZ and EMMERLING, 1990). The possible significance of such myelination may lie in the conduction of nerve impulses. Also the efferent olivocochlear bundles which make synapses with hair cells and the cochlear ganglion neuronal soma are unmyelinated (THIERS, BURGESS and NADOL, 2000). Thus some of the fibres coursing through the ganglion might not be showing any evidence of myelination. The conduction rate in human is slow which may be due to the fact that majority of the human spiral ganglion neurons are unmyelinated (CAROL and KIMURA, 1980) , very few neurons varying from 0.3-0.5% , are surrounded by loose myelin coat in young human adults (ARNOLD, 1987).

The neuronal processes and the nerve fibers showed first evidence of definite myelination at 20th week of gestation, which is in concurrence with a previous study on development of cochlear nerve myelination observed by 20th week of gestation (RAY, ROY, WADHWA et al., 2005). Sánchez del Rey, Sánchez Fernández, Martínez Ibarguen et al. (1995) believed that the empty area surrounding neurons may correspond to myelin. In the current study myelination around the neuronal soma was not evident but the presence of a few supporting cells in approximation to the neuronal soma and a bluish hue around few neurons could be an evidence of myelination in these neurons. According to literature this finding though not supported with any clear evidence till now. This could be an evidence of myelin around the perikarya laid down by the supporting cell.

4.3 Synaptogenesis

Significant neural developments in the cochlea include the acquisition of mature synaptic functions. Strong Synaptophysin immunoreactivity is found in almost all Spiral ganglion neurons of adult human cochlea (ANDERSEN, TYLSTEDT, KINNEFORS et al., 2000). Enpassant synapses and axo-somatic synapses observed in adult primates (KIMURA, OTA and TAKAHASHI, 1979; KIMURA, BONGIORNO and IVERSION, 1987; NADOL, 1988) and newborn human (ARNOLD, 1982) has been thought to represent efferent connections with the olivocochlear fibres of intraganglionic spiral bundle.

Expression of synaptophysin varies in the central nervous system. In developing spinal cord and brainstem synaptophysin immunoreaction was seen by 12-14weeks of gestation using thermal intensification technique (SARANAT and BORN, 1999). In the present study the first evidence of synaptophysin expression in cochlear ganglion was at 20th weeks of gestation which is approximately corresponding with that seen in cerebrum. Thus it can be assumed that synaptohysin is expressed almost during the same time frame in the auditory cortex and in the cochlear ganglion.

In our study synaptophysin immunoreactivity grew stronger with increasing period of gestation and was well established at 24th weeks of gestation explaining that synaptic maturation occurred before birth. Comparable studies in rat showed that synaptophysin positive fibre and terminals were seen at postnatal day3-6 (GIL-LOYZAGA and PUJOL, 1988). While in mouse at 9-10 postnatal day (SOBKOWICZ, SLAPNICK and AUGUST, 2003). Hence, lower animals respond to external auditory stimuli only after birth. While in developing human fetuses the acquisition of synapses starts in second trimester and is gradually completed well before birth as evidenced by this study.

Hence in the present study sufficient maturity of the cochlear ganglion was achieved by 24th week of gestation in terms of morphogenesis, synaptogenesis and myelination. These findings reaffirms that cochlear ganglion, the cell body of the cochlear nerve was sufficiently mature by 28th week of gestation. Hearing is thus established by the start of the third trimester. This result correlates well with the investigation of fetal audition done through the use of high resolution ultrasound imaging for observing auropalpebral reflex to a specific vibroacoustic stimulus pattern. Responses were first elicited between 24 and 25th week of gestation and were consistently present after 28 weeks (BIRNHOLZ and BENACERRAF, 1983) The cochlear ganglion attains morphological maturation and functional maturation by 28 weeks of gestation.

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