

# **Free excitatory postsynaptic densities: Morphological substrates for synapse formation and evidence of synapse elimination**

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## **Abstract**

Nerve cells communicate primarily through chemical synapses. Study of brain development essentially consists of study of synapse formation. The role played by prospective pre-and postsynaptic processes in synaptic development is not fully understood. The present study was aimed at understanding the role of postsynaptic component is synaptogenesis. This study has been carried out on prenatal (13 to 35 weeks) human lateral geniculate nuclei (LGN) immersion fixed in Karnovsky's fixative and processed for transmission electron microscopy. It was observed that the period between 16 to 20 weeks of gestation is marked by occurrence of numerous, pleomorphic free postsynaptic densities (PSDs). These free sites were located on different parts of differentiating neurons. Many of them had no prospective presynaptic partners in their vicinity, while others revealed variable amount of incongru-ency with their presynaptic partners and in some occasions they were associated with degenerating profiles. This period also coincided with many other events previously reported in the developing human LGN, e.g., spurt in volumetric growth, glial cell differentiation, synaptogenesis, cytoarchitectonic lamination as well as neurochemical maturation. It was concluded that the free PSDs are heterogenous in origin, being programmed/de novo and waiting to make synaptic contact for the first time; and vacated, the left out sites after pre-synaptic terminal degeneration and waiting to establish new contact for the second time and that glial cells play important roles in the whole process of synaptogenesis.

Introduction

Most central synapses utilize neurotransmitters for interneuronal communication and are therefore called chemical synapses. They are of two types: 1) excitatory (type I, asymmetric, or glutamatergic) and 2) inhibitory (type II, symmetric or GABAergic). The presynaptic compartment of a chemical synapse is characterized by presence of synaptic vesicles and active zone (AZ) (an electron-dense presynaptic membrane where synaptic vesicles fuse) while the postsynaptic compartment is characterized by PSD—an electron-dense thickening juxtaposed to AZ. The excitatory synapse differs from inhibitory synapse in many ways including requirement of extrinsic trophic factors during its development [1], presence of neuroligin 1 [2], and leaving free PSD after deafferentation.

Synaptogenesis is a multistage dynamic process and its each stage involves specific set of molecules for its elegant execution. Many neuron-specific molecules like synapsins [3,4] and neuroligin [5] are preferentially associated with the presynaptic terminal while others like PSD-95 [6], neuroligin [2,7,5]; and synapsin III [8] with the post-synaptic terminal. Recently, many studies have stressed on the role of glia and glia-secreted soluble factors in synaptogenesis [9,10,11,12].

During early stage of axo-dendritic synapse development, the axonal process (prospective presynaptic terminal) comes into contact with the dendritic process (prospective postsynaptic terminal) and makes a temporary contact (nascent synapse) which subsequently either gets eliminated or stabilized into a mature and functionally active connection. A nascent synapse is most likely to be a moving target – a continuum of transitional structures rather than single entity and therefore, pose difficulty in their identification with confidence, more so because the appearance of PSD differs with the technique used for its visualization [13]. PSD is an integral part of the postsynaptic signaling machinery. It organizes diverse structural and regulatory components into an elegant macromolecular assembly and within this may lie the clues to the mechanisms of the most intriguing of brain functions, including learning and memory. Therefore, a large number of studies have focused attention to unravel its molecular structure [6,2,14,5] and its possible roles in synaptic development and plasticity [5]. Though there is rapid formation and remodeling of PSD [15], and that the assembly of PSD is fundamentally different from that of the AZ assembly [16], temporal order of assembly of pre- and post-synaptic compartment has drawn sparse attention [17]. Free PSDs have been reported in adults after experimental deafferentation in the visual system [18], cerebellum [19] and septal nuclei [20] and in hippocampus during estrous cycle [9]. However, their occurrence in abundance during critical period of prenatal development [21] has not drawn much attention and hence remains an unresolved issue. Therefore, the present study was aimed at examining these free PSDs for their possible nature, role and fate during synaptogenesis as well as to seek correlation with other reported relevant events including glial cell differentiation in the establishment of interneuronal contacts.

## **Material and Method**

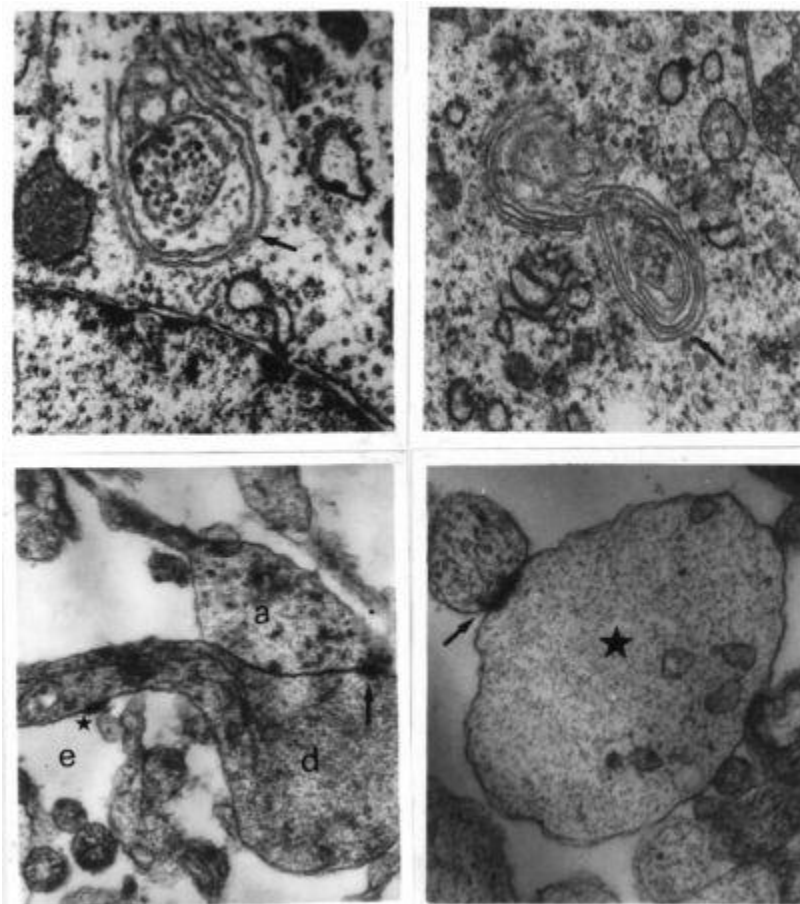
The study was conducted on human fetuses with prior permission from an ethics committee and consent of the parents. A total of 21 fetuses of both sexes ranged in gestational ages from 13-14 to 34-35 weeks were included in this study. The fetuses were

collected from hysterotomies (13-14 to 20-21 weeks) or from autopsies (25-26 and 34-35 weeks) where foetal death occurred due to spontaneous abortion. The LGN were dissected out and immersion fixed in Karnovsky's fixative for a period of two weeks and processed for araldite embedding. Ultrathin silver grey (70-80 nm) sections stained with uranyl acetate and lead citrate were used for transmission electron microscopic observations. Foetal data is given in our earlier publications [22,21].

## Observations

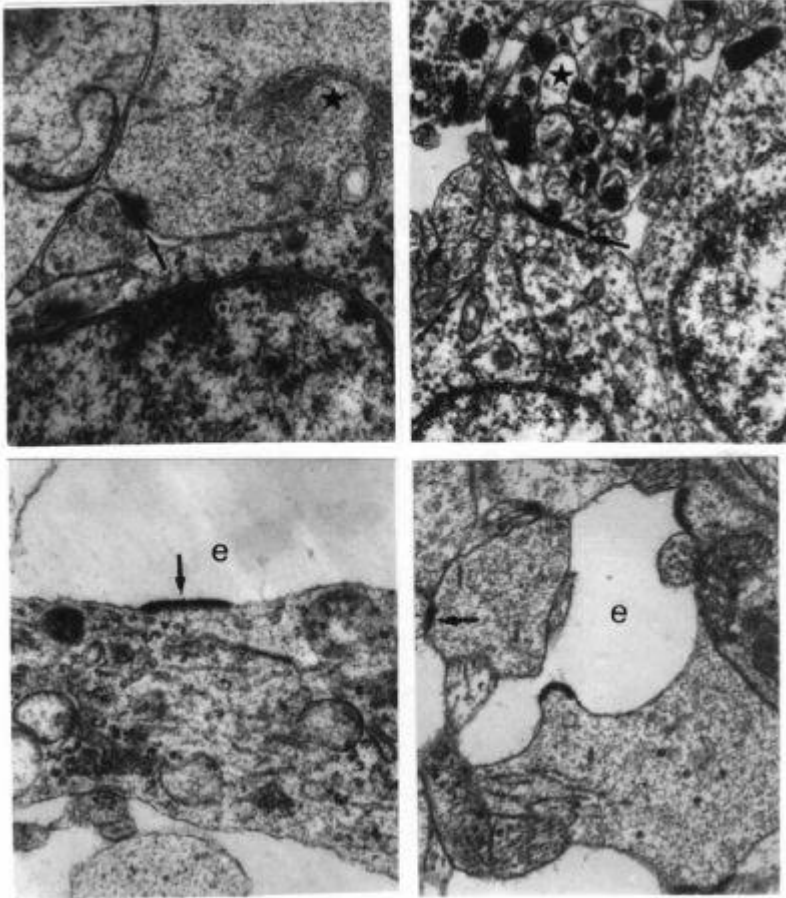
Cell and neuropil maturation. At early age of 13-14 weeks, cells are immature and both neurons and glia look alike. Only by the age of 16-18 weeks glial cells may be identified. The neuronal cell bodies during 16-21 weeks reveal tubulovesicular whirls and convolutions of membranes (Fig. 1A, 1B). Neuropil had multiple growth cones (GCs) containing pleomorphic growth vesicles, free membrane densities and frequent synaptic contacts at different stage of maturation (Fig. 1C, 1D). By 20-21 weeks the neuropil is more packed and many mature synapses (Fig. 2A) as well as some degenerating synapses (Fig. 2B) on dendrites (Fig. 2C, 2D) and soma and synaptic contacts are quite frequent. GCs make multiple contacts. The PSDs remained intact even in samples obtained from autopsy performed within 4 hours of death.

Fig. 1: Electronmicrographs from prenatal human LGN showing cytoplasmic organelles and growth cones making synaptic contacts. A) Neuronal soma at 17-18 weeks showing tubulovesicular convolutions (↑) suggestive of Golgi body with TGN-organelles at its centre. X 27953. B) Neuronal soma at 20-21 weeks showing two Golgi bodies placed side by side making a figure of δ or 8 (↑) and their tubulovesicular arrangements making half and complete rings with TGN-organelles at their centre. C) Neuropil at 15-16 weeks showing axonal (a) and dendritic (d) growth cones making a nascent synapse (↑). A free membrane density (\*) on dendritic process and prominent extracellular space (e) are also seen x16565. D) Neuropil at 16-17 weeks showing an immature axodendritic synapse (↑) between an axonal process with few synaptic vesicles and a dendritic growth cone (\*).



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Fig. 2: Electronmicrographs from prenatal human LGN showing mature synapse, degenerating synapse and free membrane densities. A) A mature axosomatic synapse (↑) at 17-18 weeks. The presynaptic terminal has many round clear synaptic vesicles and the PSD shows subsynaptic web. Soma is marked by presence of Golgi body (\*). x 20706. B) An axo-somatic synapse at 20-21 weeks with electron dense degeneration of presynaptic terminal (\*) and obliterated synaptic cleft (↑). x 20706. C) A dendrite at 16-17 weeks with robust, straight free postsynaptic density (↑) facing prominent extracellular space (e). x 27953. D) Neuropil at 16-17 weeks showing one immature synapse (↑) and two free PSDs with their outer convex surfaces facing the extracellular space (e). x 20706.



(For larger image, click [here](#))

**Synaptic contacts:** At 13-14 weeks the synapses are only few, simple, immature and exclusively asymmetric (excitatory) in nature. By the age period of 16-18 weeks growth cones and excitatory synapses are found in abundance. Some of them reveal subsynaptic web as well (Fig. 2A). By 20-21 weeks the additional features noticed are axosomatic synapses, synaptic triads and increased incidence of free PSDs. At 25-26 weeks and onwards, though the frequencies of complex synapses increased but somatic synapses as well as free PSDs are infrequent.

**Free PSDs:** Morphologically, these resemble the PSDs of a classical asymmetrical, (excitatory) chemical synapse but remain unaccompanied with appropriate presynaptic profiles (Fig 2C, 2D). These PSDs are focal, welldefined, electron dense membrane specializations. They vary in their forms, sizes and locations, i.e., both on the soma and dendrites as well as on GCs. Some PSDs are in apposition with one or more profiles (prospective presynaptic/ otherwise), variably incongruent with each other (Fig.2D). They are also located in isolated areas with no other profiles in their vicinity (Fig. 1C, 2C, 2D). Occasionally, PSDs are found to be associated with degenerating pre-synaptic terminals (Fig 2B). The cross sectional contours of PSDs vary from being straight (Fig. 2C), wavy, convex (Fig. 2D), concave, and concavoconvex. Some elaborate features like

subsynaptic web in relation to the PSD were not observed. The presynaptic profiles (committed/ otherwise), reveal variable features with respect to its congruency with PSD, membrane specializations; number of vesicle present and their location with respect to AZ.

## Discussions

The present study is a part of the work conducted on the developing human visual system dealing with its cytoarchitectural, and neurochemical maturation as well as synaptogenesis in LGN. Like other parts of nervous system, the development of LGN involves both progressive and regressive events. Synaptogenesis is a highly dynamic process. Its beginning is heralded by formation of a nascent synapse when prospective pre and postsynaptic elements come together. The retinal afferents enter the human LGN by 7-8 weeks of gestation [23]. The first retinogeniculate synapse appears at 13-14 weeks [18, 24]. Gliocytes differentiate by 16-17 weeks [22], spurt in synaptogenesis between 15-20 weeks [21] and cytoarchitectonic lamination at 20-21 weeks [25, 22]. Like many other brain regions [26] LGN also shows critical period in synaptogenesis [21], which is 15-20 weeks and is marked by concurrence of spurt in its volumetric growth [21], peak afferent inputs [27,28], substance P positive fibres [29], and GABA positive neurons [30].

The peculiar membranous arrangements (Fig 1A, 1B), also noticed in the neuronal soma around 16-18 weeks [21] were thought to be associated with high protein turn over [24]. However, a fresh look on these structures in the light of current literature [31] suggests that in fact, quite few of them are trans-Golgi network (TGN) which has been shown to carry proteins both for PSD and AZ at site of development of new synapse. As the last station of the Golgi complex, the TGN plays a pivotal role in directing proteins in the secretory pathways to the appropriate cellular destination [32]. GCs are specialized sensory structures located at the tip of neurites. In the present study GCs were found in abundance between 15-21 weeks. This period is marked by spurt in synaptogenesis which requires active growth of neurites towards their prospective synaptic partners. Synapsin III concentrated in GCs plays a role in axonal elongation [8] and interaction of actin and myosin filaments [33] and actin polymerization and de-polymerization act as force generating system [34]. Studies seeking dynamic relationship between dendritic growth and synaptogenesis suggest a 'synaptotropic model' in which synapse formation can direct dendritic arborization [35]. That is, functionally viable synapses determine the branching pattern of dendrites.

Differentiation of PSD region appears to be a prerequisite for development of synapse. Neuroligin 1 is clustered in the PSD [2], can alone stimulate formation of presynaptic specializations [36, 37] and neuroligin-neurexin link triggers synapse formation [7]. PSD in mature synapse is a complex organelle directing various cellular functions in order to integrate synaptic physiology. Although a major proportion of PSD may appear and disappear within few minutes [15], even in immature synapses it is highly organized [38]. In the present study it is most likely that the high frequency of free PSDs during 16- 20 weeks of gestation might have resulted by two mechanisms: 1) As a reminiscent of fully

developed synapses, in which the presynaptic terminal degenerated (vacated PSDs). And, possibly due to prompt handling of degeneration debris by glia, the high incidence of free PSDs was not associated with comparable incidence of degenerating terminals. And 2) PSDs formed de novo as a part of general synaptogenic process (programmed PSDs), as they were observed even in the total absence of presynaptic profile in their vicinity. The dual nature of free PSDs has also been suggested in case of experimental deafferentation of cerebellum [19]. Though morphologically, all PSDs look almost alike as electron dense structures, structurally they may be different with respect to laminar organization of their various PSD protein constituents [39, 40, 41, 42, 43], with possibility of further change in response to neural activity to match the identities of their axonal counterparts [44]. Latest studies on the measurement of mass of PSD and enumeration of key molecules [45], suggest that meticulous morphological analysis of synapses under TEM holds promise to give insight into the evolution of receptors composition in the PSD during development.

Until very recently, the role of glia in synaptogenesis remained speculative, but now the picture is becoming clearer. In developing human LGN the glia differentiate by 16-17 weeks of gestation with spurt in synaptogenesis at 16-18 weeks [21] indicating concurrence in two events. A revisit to these observations in the light of current literature, for instance, association between astrocytic processes and estradiol levels [46], steroiddependent synapse turnover and incidence of free PSD [9] appears interesting. Observation in the present study is also in agreement with the one [11] suggesting that in vivo, most synapses are generated concurrently with development of glia and that they appear to induce and stabilize the plastic and immature synapse. Now, many gliaderived molecules for example, neuroligin [10], cholesterol [47, 48], thrombospondin [12] and neurosteroids [49] are known to play important roles in synaptogenesis and neuronal circuit maturation. Thus, it underlines the significance of concurrent study on glia to make the study on various parameters of neurons more meaningful.

There is increasing evidence of roles played by neurotransmitters and neurotrophic factors in synaptogenesis. The observation of accelerated pace of synaptogenesis during 16-18 weeks is consistent with large number of substance P positive afferents [29], and GABA immunopositive neurons [30]. This assumed significance due to dual role of GABA [50] and nitric oxide [51] as synaptic transmitter and promoter of synaptogenesis. Moreover, in contrast to mature brain, where GABA is the major inhibitory neurotransmitter, in developing brain GABA can be excitatory, and glutamate may play inhibitory role in modulating the calcium elevating actions of GABA that may affect many stages of neuronal differentiation including synaptogenesis. Role of insulin [52] in promoting function of silent synapses, and those of progesterone [53] and neurosteroids [49] in neuronal circuit maturation via autocrine and /or paracrine action underline the significance of hormonal profile during development nervous system. Recent studies [54] have implicated neuroligins in mental retardation and PSD-95 in pain modulation [55], learning and addiction.

It is concluded that both pre-and post-synaptic profiles provide indispensable and interdependent contribution in the formation of mature synapse. There are at least two

subsets of PSDs namely programmed and vacated but in the absence of suitable presynaptic profiles and specific immunolabelling their distinction and quantification is rather difficult. In case these PSDs fail to find suitable partners they possibly disappear after a waiting period of few weeks. Glial cells play important roles throughout the whole process of synaptogenesis.

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