Cellular and Molecular Biology™ **56**, OL1410-OL1426
DOI 10.1170/160

ISSN 1165-158X
2010 Cell. Mol. Biol.™

INTERLAMINAR DIFFERENCES IN THE PYRAMIDAL CELL PHENOTYPE IN PARIETAL CORTEX OF AN INDIAN BAT, *CYNOPTERUS SPHINX*

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Received September 2nd, 2010; Accepted September 9th, 2010; Published October 30th, 2010

Abstract – To study interlaminar phenotypic variations in the pyramidal neurons of parietal isocortex in bat (*Cynopterus sphinx*), Golgi and Nissl methods have been employed. The parietal isocortex is relatively thin in the bat as compared to prototheria with layer III, V and VI accounting for more than two-thirds of total cortical thickness. Thick cell free layer I and thinnest accentuated layer II are quite in connotation with other chiropterids. Poor demarcation of layer III/IV in the present study is also in connotation with primitive eutherian mammal (i.e. prototherian) and other chiropterids. Most of the pyramidal cells in the different layers of the parietal isocortex are of typical type as seen in other eutherians but differ significantly in terms of soma shape and size, extent of dendritic arbor, diameter of dendrites and spine density. Percentage of pyramidal neurons, diameter of apical dendrite and spine density on apical dendrite appear to follow an increasing trend from primitive to advanced mammals; but extent of dendrites are probably governed by the specific life patterns of these mammals. It is thus concluded that 'typical' pyramidal neurons in parietal isocortex are similar in therians but different from those in prototherians. It is possible that these cells might have arisen among early eutherians after divergence from prototherian stock.

Key words: Neocortex, Extraverted pyramidal neurons, Eutheria, Interlaminar differences, dendritic spines.

INTRODUCTION

The pyramidal neurons, the sole output and the largest input system of neocortex, represent the end stage of a progressive evolutionary process. These neurons represent not only the principal neocortical elements, but also the source of various excitatory local circuit neurons [77].

Within mammals, morphology of pyramidal neuron has been studied in prototheria [48], metatherian [113] and several orders of eutheria e.g., insectivores [33, 109, 115], chiropterids [33, 35, 39, 84] rodents [1, 11, 12, 35, 41, 46, 53, 61, 74, 80, 88, 94, 96, 101, 102, 105, 115, 116,118], lagomorphs [35, 69, 71, 88, 114], artiodactyls [36, 51, 88], carnivores [15, 36, 55, 70, 82, 88, 118, 119], cetaceans [42, 44, 51, 75] and primates [16, 20,29, 30, 36, 60, 72, 83, 87, 93, 95, 103]. Thus, the present data will be an addition to phenotypic study of pyramidal neurons and differences in phenotypes of these neurons among intracortical layers of a region of a eutherian species.

Several separate studies have been done on the parietal lobe (somatosensory cortex) and morphology of pyramidal neurons. For example, findings of Ferrer [33], in the sixth layer of bat revealed a similar type of cells (pyramidal-, multiapical pyramidal-, inverted pyramids, fusiform-, horizontal-, horizontal pyramidal cells, fan shaped- Martinotti cells, spinous multipolar-, sparsely spinous- and aspinous multipolar- and bitufted- neurons) as seen in other three lissencephalic species belonging to rodentia, lagomorpha, insectivore; while in case of mustached bat [39], although cell types found in its auditory cortex were broadly similar to those described in sensory cortex of other species. However, some differences with other species were observed in terms of the laminar

Abbreviations: **ad**, apical dendrite; **bd**, basal dendrite; **ax**, axon; **wm**, white matter; **pm**, pia matter; **n**, number of visible spines; **N**, ~~esti~~mated number of spines.

distributions of the different cell types. Hassiotis and Ashwell [48] reported that percentage of pyramidal neurons in somatosensory, visual, frontal and motor cortex of a monotreme is quite low as compared to rodent. Within an order itself, the cortical pyramidal neurons exhibit different characters viz. more branching and spine density, which probably sample more inputs and compartmentalize these inputs to a greater extent in prefrontal cortex of human and macaque monkey than those in other cortical regions [19, 21-23, 56, 57]. Chen et al [11] in his study on mouse barrel cortex, stated that layer VI of the cortical system was found to be different from that of other mammals, such as felines [82] and primates [4].

Cortical pyramidal cells show marked regional as well as interlaminar differences in their phenotype which are believed to influence their functional characteristics [1, 11, 18, 25-30, 57, 65, 68, 82, 87, 103] viz. pyramidal neurons exhibit a differential distribution among cortical layers and regions, and some of them are differentially represented among species [50]. Pyramidal cells in prefrontal cortex of new world monkey are relatively simple in structure while inferotemporal cells are highly branched and spinous [23].

In addition to pyramidal neurons, several neurons have been reported as 'deviated' pyramidal neurons from 'typical' one called as atypical, [48, 77] extraverted [33, 89, 91, 108-110] or 'modified' pyramids [39].

Dendritic spines, a characteristic feature of typical mammalian pyramidal neurons, represent important structural specializations of eutherian isocortical neurons that provide most of the post-synaptic sites of axon terminating upon pyramidal neurons [31, 77]. Differences in pyramidal neuron spine density could reflect important functional differences in the isocortex [48]. Significant differences in spine density of pyramidal neurons were found between somatosensory and motor cortex of echidna and rat [48]; and visual areas in marmoset monkey [29].

These investigations show that differences do exist among pyramidal neurons in different species and in different layers of mammalian isocortex. Only a few Golgi studies exist on the morphology of pyramidal neuron in bat. The previous findings on bat are mostly related to auditory cortex that has its importance due to echolocation in bat. We decided to analyze, in first instance, the comparative analysis of several parameters of pyramidal neurons viz. relative abundance, soma shape, soma size, dendritic tree shape and size, axon length, diameter of dendrites, spine density and morphology of different layers of parietal isocortex which has its importance in integrating sensory information from different modalities; it comprises somatosensory cortex and the dorsal stream of the visual system [3]. The parietal region of bat is almost completely responsive to acoustic stimuli and occupied by the dorsal field of the auditory cortex (AC) [84]. The main objective of the present study is to present a detailed explanation of interlaminar differences in phenotypes of pyramidal neurons in parietal lobe and further comparison of these data with other earlier reported mammalian orders to find out if there is any difference in the pyramidal neurons of parietal isocortex and other areas of isocortex in mammals.

MATERIALS AND METHODS

Animal

Three bats, *Cynopterus sphinx* (Vahl, 1797) used in this study were obtained from wild in the surroundings of Allahabad (25° 28′N, 81° 54′E) (U.P., India) during March-April (25° - 36°C) and were kept in terraria until subjected to experiment. The protocol for these experiments was approved by the Animal Ethical Committee of the University of Allahabad. Male adult animals (bat) weighing 54-56 gm were used. Animals were perfused intracardially with physiological saline followed by a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (4°C, pH 7.4). The brains were removed from skulls and were left in the fixative solution overnight at 40C, then sectioned in plane normal to pial surface.

Small blocks 5 mm thick were removed from both hemispheres of the animals' brain for Golgi analysis. The left hemisphere was used for analysis by the rapid Golgi method used by Valverde [107], and the right hemisphere was used for analysis by the Golgi Colonnier procedure [2].

Nissl (Cresyl-violet) method

The brain of further one animal (already fixed) was available for cytoarchitectural study. The brain had been sectioned coronally at a thickness of 10 µm with the aid of rotatory microtome and the sections were stained with cresyl violet for the purpose of identifying cortical layers and cytoarchitectural features in the isocortical region used for the Golgi analysis. The anatomical boundaries of the parietal cortex in this study are based on the basis of the central sulcus separating the parietal lobe from frontal lobe.

Golgi-Colonnier method

The cortical pieces from the above-mentioned region were prechromed twice in 2.5% potassium dichromate for 60 min. each treatment. The blocks were then kept in a 5% glutaraldehyde v/v and 2% potassium dichromate w/v solution at 4°C for 3 days for chroming, before being transferred to a 0.75% w/v solution of silver nitrate at 40C for two days impregnation. Both chromation and impregnation steps were repeated twice, with all blocks

washed in distilled water between solutions. After the completion of the third and final impregnation, blocks were dehydrated by two 5 min. immersion in 100% alcohol, before being shelled by paraffin wax (paraffin wax, congealing point 58°-60°C, obtained from Science Corporation, Allahabad).

Sections of 180 µm thickness were cut normal to the pial surface with the aid of a sliding microtome and dehydrated in two stages of 100% alcohol for 5 min. before being washed in two stages of xylene for 5 min. each. Sections were mounted and cover slipped using DPX mounting medium.

Valverde's modification of the Rapid –Golgi technique

Cortical pieces, 5 mm in thickness, were immersed in an aqueous solution containing 2.33% w/w potassium dichromate and 0.19% w/w osmium tetroxide. The chromating solution was made by dissolving 12 gm. of potassium dichromate and 1gm. of osmium tetroxide in 500 ml of distilled water [107]; and was stored at 4°C for several weeks. A minimum of 20 ml of this solution was used for each piece. The tissue was kept in dark at room temperature for seven days. After chromation, the pieces were rinsed briefly in a small volume of 0.75% aqueous silver nitrate and stored for 24 h in a fresh volume of 0.75% silver nitrate. For each piece, 20 ml of the solution was used. The steps of chromation and slivering were repeated a further two times, with progressive lengthening of the silvering time and reduction of the chromation time. Between each step, blocks were blotted with tissue paper.

The pieces were dehydrated in two changes of absolute alcohol for 5 min. each and embedded in paraffin wax. Sections were cut with the aid of a sliding microtome at a thickness of 180 µm before being mounted on glass slides and cover slipped using DPX mounting medium.

Data analysis

Well impregnated neurons were viewed using a high magnification objective (40X). Neurons were drawn with the aid of a camera lucida attached to an Olympus BHS microscope. Cells were photographed with the help of a computer-aided microscope, a Nikon Eclipse 80i (Software, ACT-1) camera.

Quantitative analysis of dendritic spine density was undertaken from the parietal cortex using the technique of Feldman and Peters [32]. Counts were made of visible spines along 25 and 10 µm lengths of apical and basal dendrites from 5 neurons per layer. Corrections were made for underestimation of spine density due to hidden spines using measurements of spine length distribution, spine head size and dendritic diameter [32]. Only dendrites lying in a plane parallel to the section were used for analysis. The dendritic diameter orthogonal to the direction of the dendrite was measured with the aid of a calibrated eyepiece grid at five points along the shaft segment under analysis and averaged. The perpendicular linear distance from the dendritic shaft surface to the distal tip of the longest dendritic spine and the average spine head diameter were also measured using the calibrated eyepiece grid.

RESULTS

One hundred and thirty pyramidal neurons were counted in parietal isocortex of the animal presently studied. Of these, thirty six neurons were selected for analysis as their entire dendritic arbors were contained within the sections.

General structure of parietal neocortex

Parietal neocortex in bat revealed a typical mammalian pattern of six layered structure, as shown in cut-out sections stained by cresyl violet (Figure 1). Total thickness of parietal isocortex ranged from 860 µm to 1,125 µm. Layer I was devoid of neuronal somata like other typical mammalian isocortices. Thickness of layer I accounted for 11.24 % -14.52% (96.67 µm - 163.33 µm). Layer II was thinnest of all the six layers and composed of packed neuronal somata. It made 4 % - 6% (35µm-66.67 µm). Layer III was relatively cell sparse as compared to layer II and IV. It occupied 14 % - 23 % (158 µm - 195 µm) of total neocortical thickness. The boundary seperating layer III from layer IV was not very much clear. Layer IV consisted of packed somata and made up 13% - 22 % (146.67 µm - 193.33 µm). Layer V was found to be thickest and comprised of large sized and scattered somata showing low cell density. This layer accounted for 26 % - 34 % (220 µm-385 µm). Layer VI was found to be second thickest layer and was made up of relatively packed somata. It comprised of 14 % -18% (120 µm-205 µm). A clear cut demarcation could not be observed between layer VI and underlying white matter (Table 1).

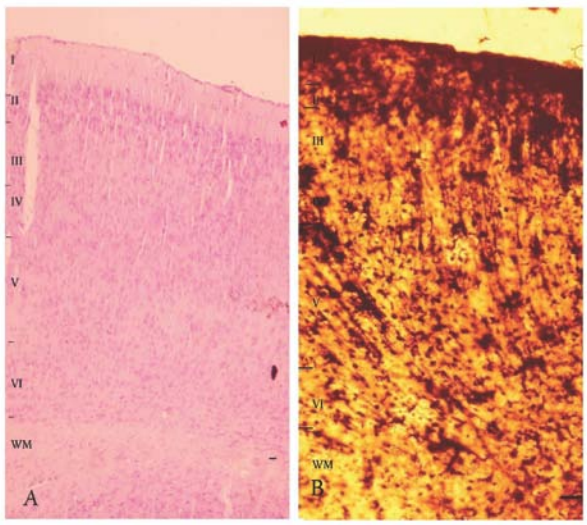

Figure 1. Cresyl-violet and Golgi stained sections through the parietal isocortex of bat. Cortical layers (I to VI) have been indicated. Scale bar = 50 µm.

Pyramidal neurons

Pyramidal neurons constituted 63.42% (130/205) of total neurons encountered in our Golgi-Colonnier preparations in parietal cortex.

Most of the pyramidal neurons were of 'classical' or 'typical' type and could be characterized by presence of : spines on their dendrites, a prominent apical dendrite running towards outer layer, a terminal bouquet of apical dendritic branches in layer I and a skirt of basal dendrites. Soma shape varied from: triangular, conical, pyriform, pentagonal, fusiform to comma shaped. There was no fixed pattern in the interlaminar distribution of pyramidal neurons as, layer V had largest pyramidal neurons, while smaller pyramidal neurons were present in outer layers II/III as well as innermost layer VI. Layer II was basically composed of extraverted neurons, making 28.57% part of total pyramidal neurons. Pyramidal neurons in parietal cortex had usually 3-5 primary dendrites originated from soma and later ramification of these into 1-9 secondary dendrites. In 'typical' cells, apical dendrites usually did not ramify or ramified quite away from soma. On the other hand, basal dendrites ramified close to soma giving off secondary and tertiary dendrites. The primary dendritic trunk had a smooth surface with few spines and generally followed a straight course after arising from the soma. The secondary and subsequent dendrites were densely covered with spines. Some spines were also observed on the distal part of the primary dendrites. The extent of apical and basal dendrites progressively increased from layer II to layer VI but the increase in vertical extension (from inner to outer layer) was more pronounced as compared to lateral extension (extension parallel to the layer).There was a considerable variance in the diameter and length of the dendrites. Cells in outer layer had smaller arbor size, while larger in inner layers. Shape of dendritic arbor was usually observed to be rounded or ellipsoidal in smaller cells, while larger cells had a longitudinally elongated or conical arbor tree shape. Axon originating from soma could be characterized by absence of spines and its vertical orientation towards deeper layers. Collaterals were not seen in the pyramidal cells of the animals presently studied (Table. 2) (Fig. 2 A, C, D, E, F and 3 A, B, D).

Some pyramidal neurons deviated from the 'typical' by showing one or more of the following features: inverted somata with apical dendrite oriented towards inner layer and bifurcation of apical dendrites close to the soma. Some of the atypical pyramidal neurons had tilted somata giving off apical dendrite which extended within same layer for considerable length, showed poorly developed basal dendritic skirt and lacked a terminal bouquet in layer I. In addition to these characters, some atypical pyramidal neurons showed some deviation in soma shape also i.e. rounded to bulb shaped somata. In addition to these neurons layer II and III (in some extent) had neurons having following characteristics- a large sized neurons presenting two oblique dendrites climbing into layer I, large sized prominent basal dendrite that reaches the deep portion of layer III and spiny; these type called extraverted neurons forms a sharply accentuated layer II. (Fig. 2B, 3C).

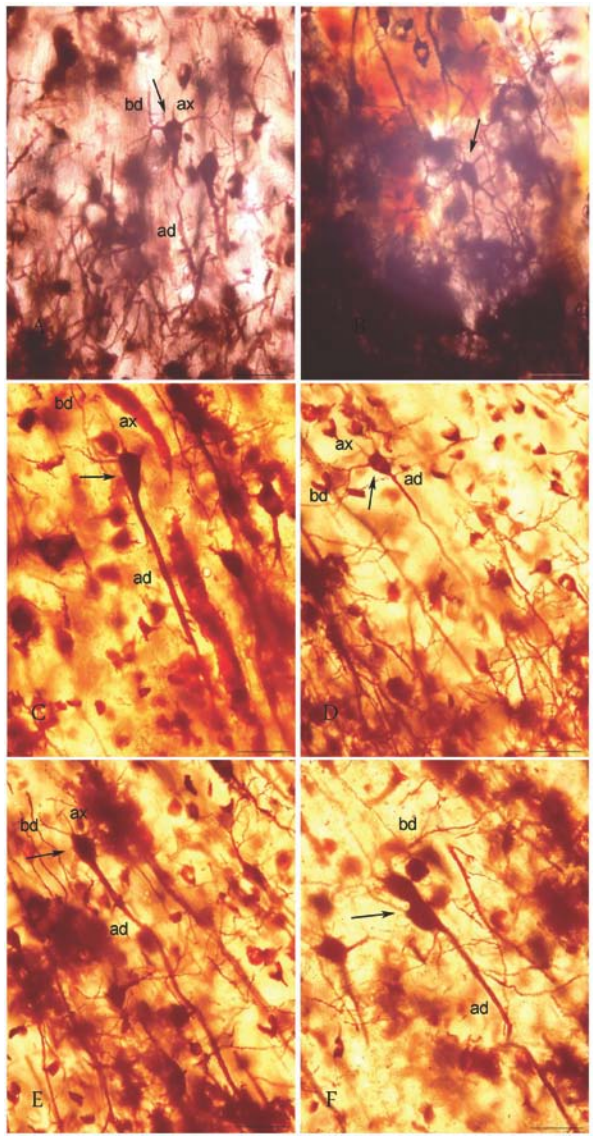

Figure 2. Typical pyramidal neurons (A,C,D,E and F) and atypical pyramidal neuron (B) encountered in bat parietal isocortex having apical dendrites (ad) running towards the pia (pm) while the basal dendrites (bd) and axon (ax) run towards the white matter (wm). Soma is indicated by arrow heads. Scale bar in D = 50 μm.

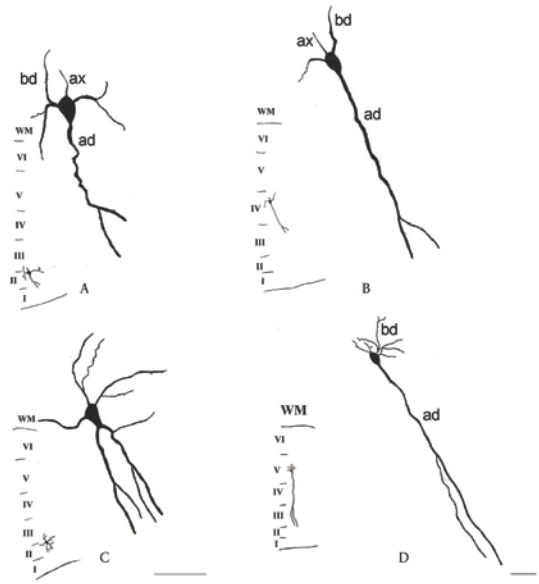

Figure 3. Camera lucida drawings of typical pyramidal neurons (A, B and D) and atypical pyramidal neuron (C) observed in parietal isocortex of bat having apical dendrites (ad) running towards the pia (pm) while the basal dendrites (bd) and axon (ax) run towards the white matter (wm). Adjoining sketches show laminar position and dendritic extent of neurons. Scale bar in C = 50 µm applies also to (A) and (B).

Percentage of pyramidal neurons

Pyramidal neurons clearly outnumbered all other classes of neuronal types viz. multipolar, bipolar, bitufted, bipolar monotufted, monotufted and unipolar in all the isocortical layers studied. Table 1 lists layer wise percentage of pyramidal neurons out of total neurons in parietal isocortex. 67.74% of neurons in layer II were of pyramidal type. In layer III, percentage of pyramidal cells was 68.75%. Layer IV showed 59.38% pyramidal cells. Layer V comprised of 63.83%, and the innermost layer VI revealed 60.32% pyramidal cells (Fig. 5A).

Atypical pyramidal neurons were frequently encountered in parietal isocortex. These accounted for 25.38 % (33/130) of total pyramidal cells. Highest fraction of atypical cells out of pyramidal cells were seen in layer II i.e. 42.86% followed by layer VI, V, III and IV i.e. 26.32%, 20%, 22.73% and 15.78% respectively (Table 1) (Fig. 5B).

Table 1. Layer width and relative frequencies of pyramidal neurons encountered in six layers of bat.

LAYERS	**LAYER WIDTH** (Total-860 µm-1,125 µm).	**TOTAL PYRAMIDAL**	**TYPICAL PYRAMIDAL**	**ATYPICAL PYRAMIDAL AND EXTRAVERTED NEURONS***
LAYER I	96.67µm-163.33 µm (11.24%-14.52% of total cortical width)	-	-	-
LAYER II	35µm -66.67 µm (4.07%-6%).	67.74%	57.14%	14.29% and 28.57%
LAYER III	158 µm - 195µm (14 % - 23%)	68.75%	77.27%	18.18% and 4.55%
LAYER IV	146.67 µm - 193.33µm (13% - 22%).	59.38%	84.21%	15.78%
LAYER V	220µm -385 µm (26%- 34 %).	63.83%	80%	20%
LAYER VI	120µm -205 µm (14%-18%).	60.32%	73.68%	26.32%

*were present only in layer II and III.

Table 2. Characteristic features of pyramidal neurons identified in bat.

CHARATERISTICS / LAYER	1.SOMA SHAPE	2.SOMA SIZE	3..PRIMARY DENDRITES	4.SECONDARY DENDRITES	5.ARBOR SIZE	6.DENDRITIC TREE SHAPE AND EXTENT	7. AXON LENGTH
LAYER II	Triangular, pyriform.	28-29µm x 10-17µm.	3-4 in no.	2-4 in no.	220-251µm x 129-132µm.	Vertically elongated to ellipsoidal. Apical -148-180µm. Basal -59-133µm.	25.64µm.
LAYER III	Triangular, rounded.	13-16µm x 11-17µm.	3-4 in no.	4-9 in no.	203-274µm x 203-217µm.	Ellipsoidal, spherical. Apical - 108-134µm. Basal -18-138µm.	80µm.
LAYER IV	Triangular, rounded, conical.	14-15µm x 13-15µm.	3-4 in no.	3-4 in no.	117-232µm x 84-161µm	Rounded, ellipsoidal. Apical -78- 134µm Basal.-83-138µm.	20.98µm.
LAYER V	Pentagonal, fusiform, bulb shaped, conical	17-29µm x 15-20µm.	3-5 in no.	3-4 in no.	359-996µm x35-161µm.	Longitudinally elongated. Apical -266-511µm. Basal -32-130µm.	20.91-78.47µm.
LAYER VI	Conical, fusiform.	15-18µm x 12-15µm.	3 in no.	1-5 in no.	179-263µm x54-187µm.	Longitudinally elongated, ellipsoidal. Apical -126-184µm. Basal -55-120µm.	18-38µm.

Table 3. Diameter of apical (ad) and basal dendrites (bd) of pyramidal neurons in bat.

LAYER / DENDRITE	II LAYER	III LAYER	IV LAYER	V LAYER	VI LAYER	ANOVA ANALYSIS			
						F value	d.f.	P<0.01	P>0.05
APICAL (mean±SD, µm)	2.04±0.92	2.04±0.47	1.83±0.60	2.122±1.23	1.64±0.24	0.59	n_1= 4 n_2= 25	Insignificant.	Insignificant.
BASAL (mean±SD, µm)	0.69±0.18	0.87±0.31	0.75±0.23	1.36±0.40	1.11±0.51	5.92	n_1= 4 n_2= 25	Significant.	Significant.

Soma shape and size

Soma shape varied from triangular, pyriform, rounded, conical, fusiform to bulb shaped.

Layer V somata were largest (17-29µm x 15-20µm) among the all the layers of parietal cortex. Layer II had second largest (28-29µm x 10-17µm) somata of parietal cortex followed by layer VI (15-18µm x 12-15µm). The smallest somata were observed in case of layer III and IV (13-16µm x 11-17µm and 14-15µm x 13-15µm) respectively. The values of layer III and IV soma size were found to be almost in same range (Table.2).

Axon

Axons in the pyramidal neurons studied were not observed to be very long and seldom were found to traverse through two layers. Length of axon observed in different layers is mentioned in Table 2. Surface of axon was

devoid of spines and sometimes appeared to be beaded. Axon in pyramidal neurons of layer II was up to 25.64µm long. In layer III, length of axon was found to be up to 80 µm. While layer IV, V, VI showed 20.98 µm, 20.91-78.47 µm and 18-38 µm long axons respectively.

Dendritic arbor size

The dendritic arbor size of pyramidal cells was found to vary considerably among the inter alia isocortical layers. (Table.2). Layer V pyramidal cells had maximum arbor size (359-996µm long x35-161µm wide). These showed maximum value of length, while layer III pyramidal cells were widest of all cortical pyramidal cells. Layer V pyramidal cells were least wide. Arbor size of layer III pyramidal neurons (203-274 µm long x 203-217 µm wide) was larger (in both the sense longer as well as wider) than layer II (220-251 µm long and 129-132 µm wide). Consequently layer II pyramidal neurons were larger than layer VI (179-263 µm long x 54-187 µm wide). The smallest arbor size of layer IV (117-232 µm long x 84-161 µm wide) pyramidal neurons was smaller than layer VI but was wider than layer V and VI.

Dendritic tree shape and extent

Dendritic tree shape varied from vertically elongated, ellipsoidal, spherical, rounded to longitudinally elongated (Table.2). Apical extent was highest in case of layer V (266µm-511µm), while lowest value was of layer IV pyramidal neurons (78µm-134µm). Layer VI pyramidal neurons had second highest apical extent (126-184 µm) followed by layer II and III (148µm-180 µm; 108µm-134µm) respectively. The highest value of basal extent was found in case of layer IV pyramidal neurons (83µm-138µm); while lowest value was of layer V and III pyramidal neurons(32µm-130µm and 18µm-138µm) respectively. Basal extent of layer II pyramidal neurons (59µm-133µm) was higher than the layer VI (55µm-120µm). (Table.2)

Diameter of apical and basal dendrite

Thickest apical and basal dendrites of pyramidal neurons found in parietal cortex were of layer V (2.12µm±1.23µm and 1.36µm±0.40µm, respectively). Thinnest apical and basal dendrites were found in case of layer VI (1.64µm±0.24µm) and layer II (0.69µm±0.18µm) respectively. The second most thickest apical dendrites were found in layer II and III (2.04µm±0.92µm and 2.04µm±0.47µm, respectively) followed by layer IV and VI (1.83µm±0.60µm and 1.64µm±0.24µm, respectively). Layer VI had second most thick basal dendrite (1.11µm±0.51µm) followed by layer III, IV and II (0.87µm±0.31µm; 0.75µm±0.23µm and 0.69µm±0.18µm) respectively. An ANOVA analysis of basal dendrite of pyramidal neurons revealed significant difference at p=0.01 (n_1=4, n_2=25), while no significant difference was found in case of thickness of apical dendrite. (Table.3)

Spine morphology

Spines present on apical and basal dendrites of pyramidal neurons were noted to be of different types. Mostly the spines were club shaped and spike-like in appearance. Mushroom shaped and elongated spines were also seen. Morphology of spines was found to be similar in neurons of all layers. Drawings of apical and basal dendritic spines found on 'typical' and 'atypical' pyramidal neurons are illustrated in figure 4.

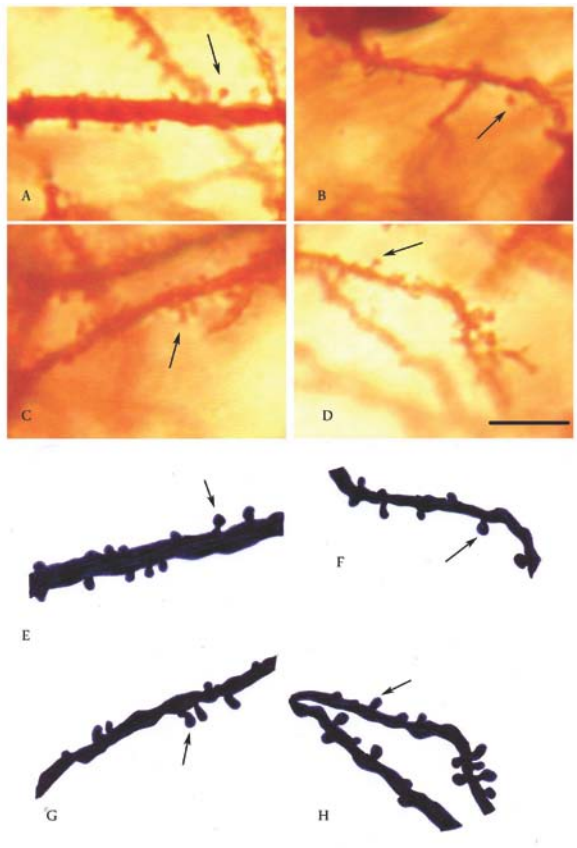

Figure 4. Photomicrographs of selected individual apical (ad) and basal dendrites (bd) of typical (A and B, respectively) and atypical (C and D, respectively) pyramidal neurons in bat (illustrating spinous processes indicated by arrows). (E) – (H) illustrate drawings of same dendrites in same order. Scale bar in (D) = 10 µm applies to (A) – (D). Scale bar in (H) = 10 µm applies to (E) – (H).

The length of spines present on apical dendrites was more than those present on basal dendrites in all layers. Table 4 shows layer wise mean spine lengths on apical and basal dendrites of pyramidal neurons. The maximum spine lengths on apical and basal dendrites were observed in layer III (2.01µm ± 0.29) and layer II (1.61µm ± 0.21) respectively; while minimum values were found in case of layer V apical and basal dendrites (1.19µm ± 0.14 and 1.13µm ± 0.30, respectively). Spines on basal dendrites of layer VI were longer than those present on their respective apical dendrite. Spine length on apical dendrites was found to be significantly different among pyramidal neurons of six layers (at p=0.05; n_1=4, n_2=25). But no significant difference was found in case of basal dendrites.

The diameter of spine head ranged from 0.63-1.52µm on apical dendrite and from 0.55-1.4µm on basal dendrites.

Spine density

The corrected spine density per 10µm of apical dendritic shaft was found to be highest in layer II (21.8±7.36), while in basal dendrite layer III (20.2 ±5.67) had highest value. Table 5 shows layer wise mean spine densities on apical and basal dendrites of pyramidal neurons. Layer III apical dendrite was the second most spinous (18.93 ±3.62), while layer IV was least spinous (15.07 ±3.45). Spine density per 10µm of apical dendritic shaft of layer V and VI was found almost similar (16.67 ±8.47 and 16.33 ±3.11, respectively). These differences revealed no significant difference by ANOVA test. In case of basal dendrite layer IV (13.33µm ± 6.14) had least spine density which progressively increased towards layer VI to II and V (14.07 ±5.45, 19.27 ±4.49 and 19.6 ±3.94, respectively). An ANOVA analysis (at p=0.05; n_1=4, n_2=25) revealed these differences to be significant. Layer II and V showed almost similar spine density (Table.5) (Fig. 5 C, D).

The corrected spine density per 10µm on dendritic shaft of atypical pyramidal neurons was found greater on apical dendritic shaft than their respective basal dendritic shaft (ap.-35.4±17.27, ba.-33.6±9.07). Table 6 shows mean spine density on atypical pyramidal neurons. Spine density on the apical and basal dendrites of atypical pyramidal neurons was found to be relatively higher than that found on the apical and basal dendrites of typical pyramidal neurons.

Table 4. Length of dendritic spines on pyramidal neurons (perpendicular distance from shaft edge).

LAYER / DENDRITE	II LAYER	III LAYER	IV LAYER	V LAYER	VI LAYER	ANOVA ANALYSIS			
						F value	d.f.	P>0.05	P>0.1
APICAL (mean±SD, µm)	1.87±0.39	2.01±0.29	1.8±0.87	1.19±0.14	1.39±0.32	3.27	$n_{1=}$ 4 n_{2} 25	Significant.	Significant.
BASAL (mean±SD, µm)	1.61±0.21	1.46±0.36	1.22±0.47	1.13±0.30	1.51±0.52	1.6	$n_{1=}$ 4 $n_{2=}$ 25	Insignificant.	Insignificant.

Table 5. Density of dendritic spines on pyramidal cell dendrites [corrected by method of Feldman and Peters, 1979].

LAYER / DENDRITE	II LAYER	III LAYER	IV LAYER	V LAYER	VI LAYER	ANOVA ANALYSIS			
						F value	d.f.	P>0.05	P>0.1
APICAL NUMBER OF VISIBLE SPINES (n) [per 10µm]	8.4	8.07	4.93	3.93	5.0				
ESTIMATED NUMBER OF SPINES ($\overline{N}$) [per 10µm]	21.8 ±7.36	18.93 ±3.62	15.07 ±3.45	16.67 ±8.47	16.33 ±3.11	1.32	$n_{1=}$ 4 $n_{2=}$ 25	Insignific-ant.	Insignific-ant.
BASAL NUMBER OF VISIBLE SPINES (n) [per 10µm]	7.33	7.33	4.93	5.87	4.27				
ESTIMATED NUMBER OF SPINES ($\overline{N}$) [per 10µm]	19.27 ±4.49	20.2 ±5.67	13.33 ±6.14	19.6 ±3.94	14.07 ±5.45	2.43	$n_{1=}$ 4 $n_{2=}$ 25	Signific-ant.	Signific-ant.

Table 6. Density of dendritic spines on atypical pyramidal cell dendrites [corrected by method of Feldman and Peters, 1979].

DENDRITE	NUMBER OF VISIBLE SPINES (n) [per 10µm]	ESTIMATED NUMBER OF SPINES ($\overline{N}$) [per 10µm]
APICAL	8.8	35.4 ±17.27
BASAL	9.6	33.6 ±9.07

Comparison of diameter of apical and basal dendrite of pyramidal neurons in bat, echidna and rat (Hassiotis and Ashwell [48]):

Diameter of apical dendrite observed in bat was compared with previously reported echidna and rat [48]. Table 7 and figure 5 (E) purport the dendritic diameter of these three mammals. It was found that echidna showed lowest value of dendritic diameter followed by bat and rat (1.76±0.37, 2.122±1.23 and 3.24±0.68). This showed a clear trend of increasing dendritic diameter from lower to higher mammals. However, this trend was not observed in case of basal dendrite, as rat showed thinnest basal dendrite closely followed by echidna (0.98±0.18 in rat and 1.04±0.26 in echidna), while bat showed thickest basal dendrite (1.36±0.4). The comparison here pertains to layer V pyramidal neurons only as dendritic diameter of only layer V pyramidal neurons has been reported in echidna and rat.

The statistical analysis, ANOVA could not be performed due to unavailability of crude observation data of echidna and rat.

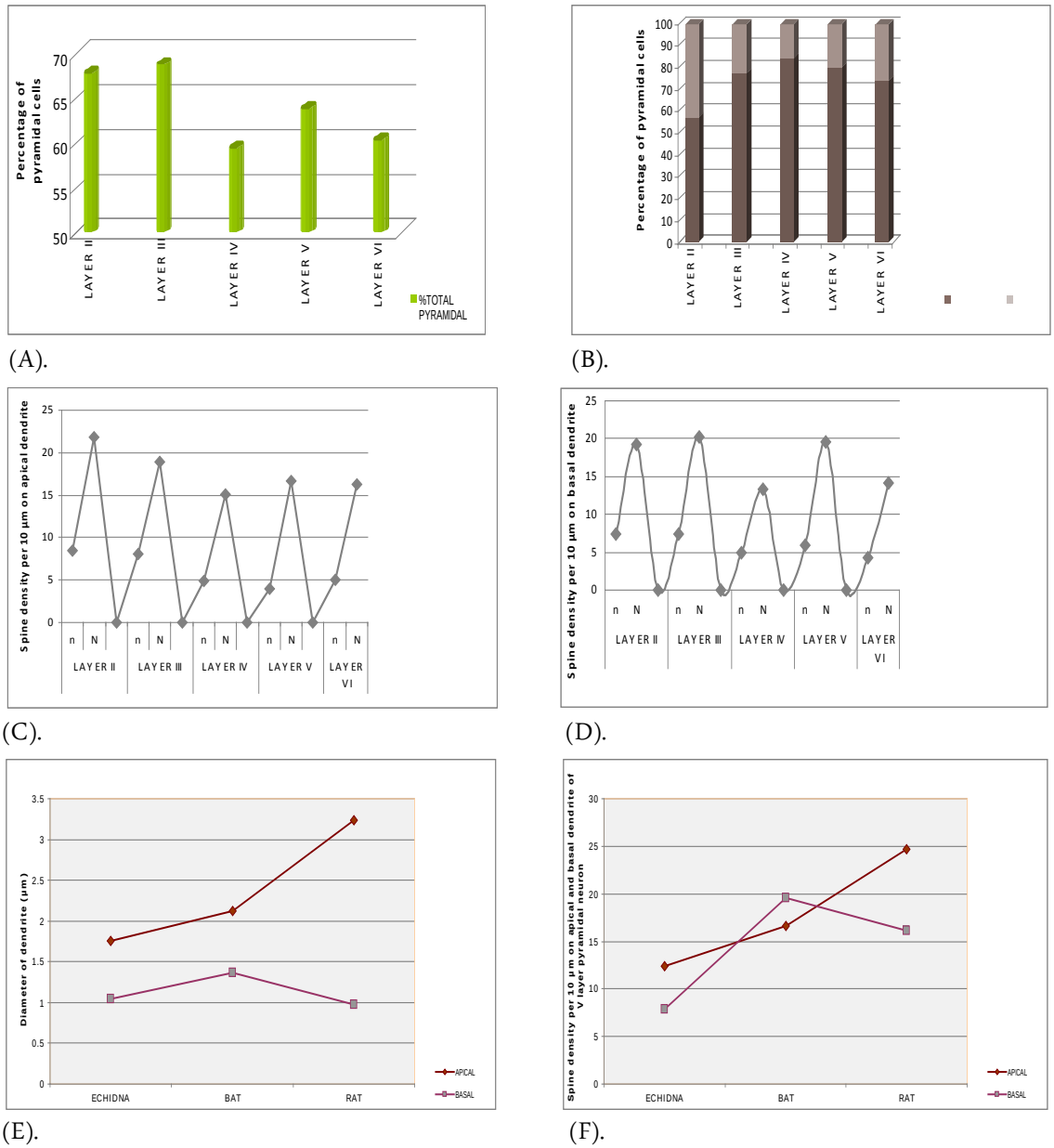

Figure 5. Plots showing different features observed in parietal isocortex of bat. (A) relative frequencies of pyramidal neurons encountered in layers II-VI of bat. (B) shows percentage of typical and atypical pyramidal neurons in layers II-VI of bat. (C) and (D) represent density of dendritic spines per 10 µm on apical dendrites (ad) and basal dendrites (bd) of typical pyramidal neurons respectively. (E) Diameter of apical (ad) and basal dendrites (bd) of layer V pyramidal neurons in bat, echidna*, and rat* (*Hassiotis and Ashwell, 2003). (F) Spine density per 10 µm of apical (ad) and basal dendrites (bd) of layer V pyramidal neurons in bat, echidna* and rat* (*Hassiotis and Ashwell'2003).

Comparison of spine density of apical and basal dendrite of pyramidal neurons in bat, echidna and rat (Hassiotis and Ashwell [48]):

Spine density on apical dendrite was lowest in echidna followed by bat and rat (12.4±3.2, 16.67±8.47 and 24.7±7.9). This comparative data also showed a clear increasing pattern of dendritic spine density on apical dendrite from lower to higher mammals. But this kind of pattern was absent in case of basal dendrite, as echidna showed least spine density followed by rat and bat (7.9±3.9, 16.1±3.8 and 19.6±3.94).

Table 8 illustrates spine density of these mammals. The comparison here again is in context of layer V pyramidal neurons only, as spine density of only layer V pyramidal neurons has been reported in echidna and rat (fig. 5F).

Table 7. Comparative chart showing diameter of apical (ad) and basal dendrites (bd) of pyramidal neurons in bat, echidna*, and rat* (*Hassiotis and Ashwell, 2003).

LAYER V DENDRITE	**ECHIDNA** (mean±SD, µm)	**BAT** (mean±SD, µm)	**RAT** (mean±SD, µm)
APICAL	1.76±0.37	2.122±1.23	3.24±0.68
BASAL	1.04±0.26	1.36±0.4	0.98±0.18

Table 8. Comparative chart showing density of dendritic spines ($\overline{N}$/10µm ± SD) on layer V pyramidal cell dendrites in bat, echidna*, and rat* (*Hassiotis and Ashwell'2003).

LAYER V DENDRITE	**ECHIDNA**	**BAT**	**RAT**
APICAL	12.4±3.2	16.67±8.47	24.7±7.9
BASAL	7.9±3.9	19.6±3.94	16.1±3.8

DISCUSSION

The present comparative data purports phenotypic variations in pyramidal neurons of the six layers of parietal cortex in bat. The pyramidal neurons form principal elements in neocortical circuitry, accounting for at least 70% of the total neocortical population. The evolutionary development of the pyramidal neurons can be traced from simple, "extraverted" neurons in the amphibian pallium, via. pyramid like neurons in the reptilian cortex [64, 98, 100] and in the hippocampus of chick, homing pigeon [104] and hippocampus and corticoid complex in strawberry finch [97, 99] to the fully developed neocortical elements designated by Cajal as "psychic cells" [77]. In present study, morphology of 'classic' or 'typical' pyramidal neurons reflected 6 principal structural properties of 'typical' pyramidal neurons in eutheria as listed by Nieuwenhuys [77], though many pyramidal neurons were found to be 'atypical' i.e. deviated from 'typical' one.

Comparison of differences found in interlaminar pyramidal neuron's phenotypes in bat parietal isocortex

General structure of parietal isocortex

In mammals, including primates, with a highly differentiated neocortex, layer I occupies only a small fraction (about 12%) of the cortical mantle [40]. However, layer I is slightly thicker (about15% to 22%) in some genus and species of bats as reported earlier [84], while in mustached bat auditory cortex [39] it comprised 17% of total cortical thickness. In present study it varied from 11% to 15%. Layer I (external plexiform layer) is an ancient superficial neuropil that has retained its basic structural organization, practically unchanged, throughout vertebrate phylogeny and ontogeny. It is similarly structured in the cerebral cortex of amphibians, reptiles and mammals. The presence within this superficial lamina of the terminal dendritic bouquets of pyramidal neurons is a universal feature recognized throughout the mammalian neocortex, paleocortex, archicortex, and intermediate cortices, as well as in the reptilian and amphibian cortices [37, 38, 49, 66, 67, 76, 86, 90, 106]. From a developmental point of view, neither the morphology of pyramidal neuron, nor the basic structural organization of the neocortex can be conceived without the participation of layer I [68]. The cetacean neocortex is characterized by a general absence of granularity, a thicker and far more cellular layer I than in most terrestrial species [52].

"External granular layer" [5] or "Layer of small pyramids" [85] i.e. Layer II, was thinnest with high packing density reflecting similarity with layer width data described earlier in mammals viz. bat [84], cetacean [42]. Layer II formed 4-6% of total in *C. sphinx* parietal cortex, while it formed 6% in mustached bat [8] auditory cortex. Layer II/III had sharp demarcation by having relatively sparse and larger cells; however demarcation of layer III/IV was not as clear in the present study. Similar findings were reported

in echidna [48] and other bats [39, 84]. Total cortical thickness of layer III and IV (combined) comprised 37% in mustached bat [39], while it was found to be 27-45% in present study. In adult dolphin, no real granular layer that might be considered homologous to layer IV was observed [42]. Layer V (26-34%) had large pyramidal cells and was most prominent & thick, as reported in most of the mammalian orders e.g., the thickest layer V in mustached bat [39] formed 24% of cortical thickness; while in dolphin, it was thinner (100-200µm) than layer III (310 µm) and layer VI (320 µm). Layer VI comprised 16% and 14-18% in mustached bat [39] and *C. sphinx* respectively. Thus, it can be inferred that most of the data related with layer width is in consistence with other mammalian orders viz. insectivores [33, 109], rodents [12, 35, 41, 80] lagomorphs [35, 69], carnivores [15, 36, 119], and primates [20, 29, 30, 51, 95] except few like monotremata [48], chiropterids [33, 35, 39, 84] and cetaceans [42, 44, 51]. Echidna and bat show poor demarcation of layer IV boundaries which could be related to their primitiveness as echidna is a primitive mammal and bat is a primitive eutherian. Thus, it can be summed up that though the somatosensory cortex is thicker in monotremata, a clear demarcation of cortical layer III/IV is not present in monotremes and primitive eutherians. This has been postulated by many workers (Sanides [89]; Sanides and Sanides [91]; Ferrer [34]; Glezer et al. [43]) in their concept of the 'initial protomammalian brain organization' that for a 'primitive' cortex, the thalamic projections terminate in layer I, which is more prominent than the underdeveloped layer IV. This finding well suited to the present study, suggesting the presence of a primitive type of neocortex.

Relative frequencies of pyramidal neurons

As reported earlier [33, 39, 42, 44, 84, 92], densely packed, 'accentuated' layer II, had 'extraverted' pyramidal cells which were about half of total neurons. These findings are comparable to layer II of the bat, *C. sphinx*. The unusual morphology of the extraverted neurons serves to receive the thalamic projections terminating in layer I in 'primitive' cortex [34, 43, 89, 91]. Layer II had total of 28.57% extraverted neurons and 57.14% typical pyramidals in the present study, while in mustached bat auditory cortex [8] it was found to be 59% and 38% respectively. Layer IV had highest number of typical pyramidal neurons followed by layer V, III, VI and II; while percentage of atypical pyramidal neurons is increasing towards inner layer (except layer IV). As stated earlier, inverted and atypically oriented pyramidal cells are present in the cortices of rats [73, 79], rabbit [45], dogs [36], cats [36], and humans [112, 117]. The inverted pyramids are one of the most common of the atypically oriented types [112], and they are more frequent in deeper layers (due to their early development) [17, 34-36, 111] and in abnormal cortex [45, 61, 81]. Atypical pyramidal neurons, characterized by bifurcated apical dendrite close to soma, tilted somata and poorly developed basal dendritic skirt, were mainly observed in layer II/III in rat [48] but, in bat (*C. sphinx*), these cells were observed in all layers which is comparable to echidna as reported by Hassiotis & Ashwell [48]. Percentage of total typical pyramidal neurons in parietal isocortex of bat was found to be 74.62% (97/130), while in somatosensory cortex of echidna; it was reported to be 67% [48]. Atypical pyramidal neurons in the echidna isocortex makes up between 30 and 42% of all pyramidal neurons, while in case of bat it was 25.38% (33/130) of all pyramidal neurons. According to Hui-Xin Qi et al. [83], study on chimpanzee sensorimotor cortex, the proportion of such cells may vary across species and cortical areas, but are generally uncommon, perhaps 1-3% (10% in layer VI), while 15 and 8.5% in layers V-VI of rabbit primary and secondary visual cortex respectively [71]. In marsupial, such as opossum (*Didelphis virginiania*), predominant cell type in cortex is pyramidal neuron [113]. Thus, it seems that pyramidal neurons are most abundant cell types in all mammals and proportion of atypical pyramidal neurons show a continous decrease from primitive to advanced one, as also supported by Nieuwenhuys [77].

Soma shape and Size

Findings on soma shape of present study is mostly similar to those of reported in other mammalian orders including prototheria, however rounded somata a characteristic feature of bat, *Pteronotus* [39] was also reported in the present study. Shape of soma in pyramidal neurons varies from conical to rectangular in echidna [48]; transitional types like quadrangular, triangular, club or mace shaped in cetacean [44] and triangular, fusiform, oval or irregular or bizarre forms which no longer appear as pyramidal neurons [72]. Probably, there is no set pattern of pyramidal neurons in mammals as

they show diverse forms. Although, rounded shaped soma has been observed mainly in bats.

Neuronal soma volume is determined by the biosynthetic and metabolic requirements of the entire cell, including its dendritic arbors and axons [59]. Similarly, evolutionary enlargement of Betz and Meynert (gigantopyramidal subtypes) somata represents increase in the thickness and/or ramifications of these 'cells' neuritis. The enlarged Betz somata might serve a role in some aspects of locomotor adaption of primates [95]. In opossum, small pyramidal cells have somata in layer II/III, medium sized in layer IV and large cells in inner layers [113]. Similar distribution has been observed in cetacean [44] visual cortex, hedgehog, elephant shrew and bat (*Myotis lucifugus*) (Sanides and Sanides [92]). The present studied bat (*C. sphinx*), also had largest somata of pyramidal neurons in layer V as reported in other mammalian orders.

Arbor size and ramification of dendrites

There are some degree of variation in the dendritic arbor size, branching pattern, spine density and somal area of neurons in each cortical area [14, 18, 23, 62, 63, 78] which reflect specializations in their structure [24, 58]. The greater number of spines on the basal dendritic fields of layer III pyramidal neurons in "higher" areas, and their more widespread dendritic trees, may allow an increased degree of association, allowing integration of different features over larger regions of cortex [23]. In the present study, number of primary dendrites of pyramidal neurons was highest in layer V, while layer III had highest number of secondary dendrites. The arbor size of layer V had highest value followed by layer III. However, layer VI had pyramidal neurons of smallest arbor size which reflected findings of Caviness [7, 8], Caviness and Sidman [9, 10] i.e. anomalous location of polymorphic and large pyramidal cells in a superficial position, while smaller neurons in successively deeper layers; and Morgane et al. [75], where very large pyramidal neurons were found at the border between layers III and V.

Dendritic tree shape and extent

Shape of dendritic tree was found to be spherical to vertically elongated which is similar to that reported in echidna [48]. A pyramidal cell is distinguished by its characteristic apical dendrite and basal dendrite arbor [13, 31]. Layer V contains large sized pyramidal neurons [103]. In present study, the highest apical extent of layer V as well as basal extent of layer II/III were found to be quite low as compared to that reported in somatosensory cortex in monotremata [48] (150 μm- 1.5 mm and 150- 200 μm, respectively). Layer VI, which according to Prieto and Winer [82] has a special role in sensory neocortex because of its diverse connections, had second highest value of apical extent in presently studied bat. Smaller dendritic extents in bat can be attributed to comparatively thin parietal isocortex than echidna.

Axon length

Each pyramidal neuron elaborates extensive intracortical axon collaterals that generate the majority of excitatory input in neighbouring cortical neurons [6]. In bat (*C. sphinx*) orientation of axons were similar to those found in other mammals (e.g. echidna [48] and mole [33]) i.e. vertical axons descending towards sub cortical white matter. However, in mole many pyramidal cells of layer V and VI had ascending axon collaterals that reached layer IV and even the inner region of layer III. Mostly, axons were less ramified or were mainly unbranched in bat, while many horizontal and oblique collateral branches were reported in the isocortex of the mole [33]. In echidna, the axons of some pyramidal neurons in layer V revealed branching but others were mainly unbranched and vertical.

Apical and basal dendritic diameter

While comparing the data on dendritic diameter with that of findings in echidna [48] motor and somatosensory cortex, in layer V apical dendrite, a clear increasing order from echidna to bat and rat was observed. Thus, by looking upon it, appearently it formed a trend in increasing thickness from primitive monotremes to advanced eutherian, but no clear pattern was observed in case of basal dendrite; while basal dendrite showed a significant difference in the thickness among six layers of bat (*C. sphinx*).

Spine morphology and spine density

Dendritic spines were mostly club shaped and spike-like in appearance. Hassiotis and Ashwell [48] reported similar morphology of spines in echidna and rat. Length of spines were also reported to be similar in rat and echidna; but, in this study, significant difference was found in spine length of apical dendrites among six layers of parietal cortex, with layer III showing longest spines. According to Harris et al. [47], large spines have large postsynaptic densities.

Diameter of spine head reported in present study has relatively higher values as compared to those reported in rat and echidna [48]. Spines present on apical and basal dendrites of atypical pyramidal neurons showed similar morphology as found in typical ones.

Horner and Arbuthnott [54], based on their study of spine density, concluded that distribution of spines over the dendritic field is not uniform but uneven. This finding is well in concordance with the present data on bat spine density which also revealed uneven distribution of spines among interlaminar dendrites of pyramidal neurons. Spine density was found to be significantly different among interlaminar basal dendrites of bat. Highest spine densities per 10 µm were observed in layer III basal dendrite, which is in accordance with Elston et al. [23]. Comparative analysis of layer V spine density in echidna [48], rat [48] and bat revealed variations among different groups/ orders. The data on spine density provided by Elston [50] also showed a significant regional variation. It is interesting to note that an increasing trend in the spine density of layer V apical dendrite from echidna to bat and rat (i.e. from primitive to advance) has been observed but no trend could be observed in basal dendritic spine density.

In an overall conclusion, it can be said that the pyramidal neurons have increased in relative abundance in mammalian isocortex as an evolutionary trend from primitive to advanced ones. Not only abundance, but, soma shape and size, extent of dendritic arbor, diameter of dendrites and spine density have evolved on separate lines followed by each mammalian order and their habit and habitat as significant differences among these characters have been observed.

Acknowledgements - We wish to thank Head, Department of Zoology, University of Allahabad, Allahabad, U.P., India for providing necessary facilities required in this study. Thanks are due to Dr. Kailash Chandra, Additional Director, Z.S.I., Jabalpur, for identification of the bat presently studied. This work is supported by UGC "Research Fellowship in Science for Meritorious Students" (order no. 05/R/111/2009) awarded to Suchi Vrata Pathak.

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