Prospective microglia and brain macrophage distribution pattern in normal rat brain shows age sensitive dispersal and stabilization with development

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The monocytic lineage cells in brain, generally speaking brain macrophage and/or microglia show some dissimilar distribution patterns and disagreement regarding their origin and onset in brain. Here, we investigated its onset and distribution/colonization pattern in normal brain with development. Primarily, early and late embryonic stages, neonate and adult brains were sectioned for routine H/E staining; a modified silver-gold staining was used for discriminating monocytic lineage cells in brain; and TEM to deliver ultramicroscopic details of these cells in brain. Immunofluorescence study with CD11b marker revealed the distribution of active microglia/macrophage like cells. Overall, in early embryonic day 12, the band of densely stained cells are found at the margin of developing ventricles and cells sprout from there dispersed towards the outer edge. However, with development, this band shrunk and the dispersion trend decreased. The deeply stained macrophage like cell population migration from outer cortex to ventricle observed highest in late embryonic days, continued with decreased amount in neonates and settled down in adult. In adult, a few blood borne macrophage like cells were observed through the vascular margins. TEM study depicted less distinguishable features of cells in brain in early embryo, whereas from late embryo to adult different neuroglial populations and microglia/macrophages showed distinctive features and organization in brain. CD11b expression showed some similarity, though not fully, with the distribution pattern depending on the differentiation/activation status of these macrophage lineage cells. This study provides some generalized spatial and temporal pattern of macrophage/microglia distribution in rat brain, and further indicates some intrigue areas that need to be addressed.

Keywords: CD11b expression, CNS, Embryo, Microglia, Silver-gold staining, TEM.

Microglia are population of blood derived monocytic lineage cells in brain. As they are mesodermal in origin and blood born, they cannot be synthesized de novo in brain where the cells are of ectodermal in origin and commonly generated from a common progenitor known as neural stem cells¹,². Therefore, these cells must have to enter in brain and colonize there in any or different phases of life. The origin of microglia and their entry into brain is still an issue of debate. Though, their mesodermal monocytic ancestry was confirmed in recent past against the claim of their ectodermal derivation, their entry and residence in brain still evokes some controversy. Conventional idea is that, monocytic lineage cells enter into brain in pre-natal phases of embryo development streaming from subarachnoid and ventricular regions, and settle down after birth as resident microglia³-⁶. In adult, resident microglia remain there and monitor the CNS environment until some pathophysiological situation signals them to some activation state⁷-⁹.

Until Djukic and co-workers¹⁰ demonstrated other possibilities in 2006, it was believed that the blood macrophage/monocytes in adult are incapable of entering into brain except during pathogenesis when blood-brain-barrier is disrupted. Still, there are several areas regarding brain macrophage/microglia entry and distribution in normal brain development and aging which can be reconstructed with greater details. Particularly, how much the microglia are the resident population in brain and from when, whether blood borne macrophage like cells populate in brain at some different time frame in life, and their morpho-functional transformation in brain microenvironment.

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are the areas to delve with critical attention. Proper understanding of these issues in normal brain tissue will help in monitoring its activation and involvement in any neuropathogenesis with greater accuracy. In the present study, we tried to ascertain the basal distribution trend of the cell with development and aging in rats of different age groups.

**Materials and Methods**

**Animal and Grouping**—The Sprague-Dawley rats were grouped into a) Embryonic day 12 (ED12); b) Embryonic day 18 (ED18); c) Neo-natal day 5 (D5); and d) Young Adults of 45 days (D45), and fed with hind liver pellet or equivalent and water *ad libitum*, maintained 12 h light and dark cycle, examined and weighed at regular intervals throughout the experimental period. They were maintained according to the animal experiment procedures strictly followed ‘the Principles of Laboratory Animal Care’ (NIH publication no. 85-23, revised in 1985) as approved by institutional ethical committee.

**Brain tissue sections and H/E staining**—The rats were deeply anaesthetized with sodium pentobarbital. The whole brain was dissected out, initially placed into ice-cold PBS buffer and finally preserved in 10% formalin-PBS. The coronal sections of brain with prominent cortex and lateral ventricular regions were processed from the brains of each group with 8-10 µm thickness. The sections were routinely stained with hematoxylin-eosin.

**Silver gold staining of brain tissue**—To stain the brain sections, the method of silver carbonate staining introduced by del Rio Hortega (1918) and gold toning by Penfield (1937) and modified by McCarter (1939) was adopted\(^\text{11}\),\(^\text{12}\). Tissue sections of 8-10 µm thickness were rinsed with 3-5 drops of strong ammoniacal aqueous solution for 1 h. Later, the sections were passed through Globus 5% hydrobromic acid solution, incubated at 37°C for 30 min followed by dH\(_2\)O wash. The washed slides were rinsed with 50% aqueous dilution of silver carbonate readily derived from reaction between silver nitrate and sodium carbonate in ammoniacal medium for 1 h and were passed through 5% formalin for 1 min with gentle shaking, washed and placed in 1% aqueous gold chloride solution for 1 h. Finally, the slides were rinsed thoroughly in dH\(_2\)O and fixed in freshly prepared 5% sodium thiosulfate solution for 2 min, washed in tap water, dehydrated in graded alcohol and mounted in DPX to observe under microscope.

**Immunofluoresence microscopy**—Paraffin embedded brain tissue sections of 8-10 µm thickness were prepared, preheated overnight at 50°C and deparaffinization was done with xylene for 2-3 min. Sections were gradually hydrated with graded alcohol and finally rinsed with distilled water for 2 min. After a PBS wash, sections were mildly fixed with 2% paraformaldehyde solution for 10 min at 24°C and the slides were immersed in 2% BSA solution for 30 min at 4°C and excess BSA was wiped out. The sections were then stained with fluorescinated anti-CD11b antibody (eBioscience, USA) diluted in 1% BSA in PBS (1:200) and incubated for 1 h in dark humified chamber. Finally, sections were washed with PBS, semidried and mounted in DPX. Immunostained sections were then viewed in Zeiss AX10 Microscope, Germany with HBO 50 lamp source and documented by Canon 1000D camera.

**Transmission electron microscopy of brain tissue**—Dissected out brain tissues were fixed in equimolar mixture of 2.5 % glutaraldehyde & 2% paraformaldehyde and kept at 4°C for overnight. The desired regions of brain, specifically, from outer boundary of cerebral cortex to ventricle, were chopped out for further processing. Tissues were treated with freshly prepared 2% OsO\(_4\) in isotonic condition for 2 h at 4°C and gradual dehydration was done in acetone, and were kept in absolute acetone for overnight, passed through dry acetone infiltration with toluene at room temperature. A mixture of toluene-resin was used with increasing resin concentration which gradually transferred to absolute resin with changes and increasing temperature up to 62°C in flat mold. By using glass knife (Leica EM KMR3), the blocks were ultra-sectioned in Leica EM UC6 to 80 nm thickness, placed in copper grid, air dried and observed under the FEI Tecnai G\(^\text{2}\) Biotwin Spirit (20-120KV), USA to identify and observe the ultra-structure of microglia in brain sections.

**Results**

**Genesis and radiation of neuroglial precursor cells in early embryo**—Staining of brain tissue of different ages with normal haematoxylin-eosin method showed nearly similar staining of different cellular components in brain and thereby the overall cellular distribution in the organ. The presumptive ventricle of early developing brain was surrounded by dense cell gathering which gradually became less dense towards
periphery (Fig. 1A). In silver-gold staining too, same arrangement of cellular distribution or radiating pattern was observed. However, a distinct band of cellular composition was clearly visible along the ventricle margin from where densely stained cells appear to be sprouting. Their cellular and staining density fed out as they approach to outer margin of developing cortex. Fig. 2A (i) & (ii) shows area close to ventricle and Fig. 2A(iii) shows area close to periphery. To the periphery, among presumptive neuroglial background, some densely stained nearly round cells could be observed of which some were possibly from early arachnoid space at the upper margin of cortex. Immunofluorescence against CD11b showed a dense gathering of CD11b+ cells in early embryo at that early arachnoid area and blood-island like vascular enclosures in the forming brain tissue (Fig. 3Ai & 3Aii). Remarkably, no such

Fig. 1—H/E staining of brain tissue of rat at different developmental stages. (A) Section of early embryos show a thick band of deeply stained cells from the margin of ventricle radiating towards periphery (10X); (B) A distinct band of cells appears along the ventricular margin in late embryo (40X); (C) In neonatal rats, different glial populations (oligodendrocytes and astrocytes), few neuronal cell bodies and prominent capillary sections with its definite lining, perivascular cells are visible (40X); (D) In adult brain, overall cellularities of brain cells are visible, but the distinction among different cell population is not clear (40X).

Fig. 2—The silver-gold staining method of brain tissue. (A) Highly populated ventricular margin of early embryonic rat brain shows neurogliogenesis and cells disperses towards outer margin of brain (Ai at 4X & Aii at 10X), whereas from outer margin certain densely stained cells scatters in the brain tissue matrix (Aiii at 10X); (B) In late embryo, densely stained cells show increase in number in outer margin of cortex (Bi at 10X) and less towards ventricular margin (Bii at 10X); (C) But in neonatal rats, these cells are evenly dispersed and considerably populated in brain matrix (Ci & Cii at 10X); (D) In young adults, these cells are dispersed in brain with round and elongated shapes among neurons and other cells (Di at 10X & Dii at 40X), and similarly stained cells along the margins of blood vasculature in brain matrix (Di); (E) In an adult of day 90, both amoeboid and ramified microglia with projections are clearly visible at 40X.
CD11b+ cells were visible at this stage in early brain parenchyma. Ultramicroscopy of tissue sections near ventricular margin showed densely stained cell nucleus with less cytoplasmic area, and nearly similar features in close proximities (Fig. 4A). These are the dividing undifferentiated neuroglial progenitors near the site of neurogliogenesis.

Colonization of myeloid lineage cells in brain in late embryo—Normal HE staining of the brain showed remarkably different cellularity near ventricle. A cellular band was distinct at the margin, but immediately after that diffused cell matrix starts with scattered cells (Fig. 1B). Silver-gold stain could differentiate the cells where probable myelomonocytes showed dark stains, but other neuroglial cells showed differential and lighter stain. Towards outer margin, these dark stained cells showed a dramatic increase in number (Fig. 2Bi), but towards ventricle they were very few in number (Fig. 2Bii) showing a gradient of their population from outer cortex to inner ventricular margin. In contrast to early embryo, the brain parenchyma in cerebral cortex of late embryo showed increased densely populated CD11b+ cells (Fig. 3Bi). Higher magnification revealed their presence in small capillary lumen at its margin to inner parenchyma as well (Fig. 3Bii). The ultrastructure of the tissue revealed that, in brain parenchyma, maturing neurons of neonates are in close attachments with these microglia cells (Fig. 4B).

Dispersal of microglia and close contact with neurons in neonates—For neonatal brain, normal H/E staining showed dispersion of cellular components. Though cellular distinction was not prominent, the blood borne monocyte/macrophage like cells were found in capillary, perivascular space and in brain parenchyma (Fig. 1C). The silver-gold staining method showed distinct variation of staining

![Image](image-url)

**Fig. 3**—Immunofluorescence microscopy for CD11b receptors shows that: (A) In early embryo nearly no CD11b+ cells in brain, but they are populated either in presumptive arachnoid space of forming brain or in some blood vasculature pools in brain matrix (Ai at 10X & Aii at 40X); (B) In late embryo, many CD11b+ cells are scattered in brain matrix (Bi at 10X), in blood vasculature crosssection and surrounding brain tissue (Bii at 40X); (C) In neonates, CD11b+ cells are scattered from outer to inner cerebral cortex, in arachnoid spaces and blood vasculatures running through brain tissue (Ci at 10X); (D) In young adults, CD11b+ cells are fewer in tissue, mostly found in blood capillary/vasculature margines (Di at 10X). In Dii, CD11b+ cell pool are found where CD11b+ cell with ramifications are found between vasculature and tissue space, depicting perivascular microglial cells (Dii at 40X).

![Image](image-url)

**Fig. 4**—Transmission Electron Microscopy (Mg=microglia, N=neurone). (A) Densely packed cells with minimum variability to the ventricular margin of early embryo showing the undifferentiated nature of neuroglial precursor cells; (B) Differentiated neuronal cells and closely associated microglial cells in the cerebral cortex of late embryos; (C) Nearly same features of neuron-microglia close association as in neonatal rats; (D) In young adult, microglia and neurones are found more distant and scattered in the brain tissue.
density for the microglia/macrophage like cells with their darker appearance than other cells. They were found dispersed among other cells both in cerebral cortex outer margin and near ventricle (Fig. 2Ci & Cii). This section showed that densely stained microglia cells were more evenly intercalated among neuroglial components with reduced density in comparison to late embryo brain and sometimes in close contact with other cells. Immunofluorescence of CD11b showed positive cells scattered in brain matrix and densely packed in blood lumen or capillary sections (Fig. 3C). Transmission electron micrograph of ultrathin sections of brain tissue revealed that many microglia and neurons were in tight attachment with each other’s. Microglia with their densely stained nucleus and cell body nearly encircled the neuronal cells with prominent cell bodies (Fig. 4C).

Spreading, stabilization and shape changes in microglia in young adult, also showing capillary escape routes—In H/E staining general decrease in cellular density of brain tissue was obvious (Fig 1D). But the nature of cells were quite indistinguishable with few vacuole like spaces. Silver-gold staining showed some elongated dark stained cells in brain parenchyma, i.e., with ramified appearance along with some dark stained round cells (Fig. 2Di & Di). This heterogeneity of microglial shapes in the vicinity of several neuronal cells was prominent (Fig. 2Dii). The ‘ramified’ microglia with their slender projections, elongated cell body and nucleus were found clearly in 3 months old rat brain (Fig. 2E). At the same time, it was observed that similarly stained cells were tethered with the margin of blood capillary or lymphoid vasculature, of which some reside on the entry into the tissue matrix (Fig. 2Dii). The immunofluorescence micrographs of CD11b+ cells showed few diffusely dispersed positive cells in the brain parenchyma, and few more either in capillary sections or vascular cavities (Fig.3Di & Dii). In the porous vascular area of brain parenchyma, a group of blood borne CD11b+ cells gathered together, where a CD11b+ cells with slender projections anchoring between vasculature wall and tissue boundary were visible in perivascular space, and another group of CD11b+ cells were found just entering into tissue matrix (Fig. 3Di). In TEM also, it was found that microglial cells were not so closely associated with neurones or other cells and scattered in tissue matrix in brain (Fig. 4D).

Discussion

Opinion on identification of exact progenitor of microglia in brain and their onset or entry into brain varies among researchers.\(^ {3,6} \) In the present study, we tried to analyze the spatial and temporal pattern of macrophage/microglia distribution in early and late embryonic stages, early postnatal and adult rats. Starting with simple H/E staining initially, we found the general cellular distribution pattern in brain. But lack of efficiency of this staining to distinguish among neuroglial populations, particularly for microglia/macrophages, influenced us to adopt different staining method. We depended on the classical silver-gold staining technique which was introduced, developed and modifies by del Rio Hortega, Penfield, McCarter and others; and used predominantly to stain brain macrophages/microglia before the development of antigen dependent staining\(^ {11,12} \). On formol reduction of silver, the sections have a golden-brown colour and other structures are slightly impregnated. However, after gold–chloride toning, colloid like gold particles substitute the metallic silver and hyposulfite fixation removes oxidized silver. This gold substitution dramatically reduces background to almost zero and the final image is formed by metallic gold. This exclusively stains the neuronal cell body and projections, oligodendrocyte nucleus, and densely stains microglia and almost negligibly, astrocytes and brain matrix, and thereby produce better clarity. Additionally, we combined the present receptor based analysis for detecting myelo-monocyte derived cells. Accordingly, we selected CD11b receptor which is mostly expressed on activated monocytes as well as macrophages in brain\(^ {11,12} \). We identified CD11b expressing cells in brain parenchyma, vasculatures etc., and tried to corroborate with S/G staining at different developmental ages. Further, with transmission electron microscopy, the ultrastructure of cell was clear with its cellular organization in different ages of brain.

Both S/G staining and CD11b immunofluorescence staining showed that at nearly 18 embryonic days blood borne monocyctic cells mostly populate in brain in huge number, and migrate mostly from outer arachnoid space of cerebral cortex and inner blood vascular lumens to different regions of brain parenchyma of rat. In few cases, the ventricle margin also appeared to have a role in this invasion as reported earlier.\(^ {13,15,16} \) But at embryonic day 12, these blood borne cells get clustered in great number around developing brain at presumptive arachnoid area and
are negligible in brain parenchyma which was then getting populated with other neuroglial cells from ventricular margins. From neonates, they were getting dispersed and acquiring their functional positions as found in proximity with neuronal cells prominently in TEM (Fig. 4C), where they can act in neuronal circuit formation and pruning at that stage of development. Our observation of microglial proximity to neurones in cerebral cortex in neonatal rat brain support the neuronal pruning function of microglia. In all cases of transmission electron micrographs, except the early embryo, microglia showed densely stained nucleus with signs of heteropyknosis, whereas neurons showed larger cell bodies and nucleus with granular spots in TEM.

Microglia continues to spread with increased brain space and morphological diversity appears from young adults. In young adult, interestingly, these blood borne cells appeared at the vascular margins and few at the entry of the brain tissue (Fig. 2Di). Similar appearance was found at the blood lumen margin for CD11b+ cells with projections in perivascular space and other adjacent to it in brain matrix (Fig. 3Dii). It suggests a continuous transition process from jux travascular or perivascular microglia to resident microglia from blood monocytes. It shows that there may be possibility of entry of monocytc lineage cells from blood to brain in adult normal brain also, though rare in occurrence. Overall, the cells showed circular morphology in both the embryonic stages and continued with the same morphology largely in neonates also (Fig. 2Ai-C). However, from neonates few elongated cells started to emerge (Fig. 2C) which increased in young adults (Fig. 2D), and some showed typically clear ramifications (Fig. 2E & Fig. 3Dii). This transformation actually shows that with dispersal and stabilization of infiltrated monocytc lineage cells in developing brain they are changing their morphology from circular (‘amoeboid/activated’) to elongated (‘ramified/resting’) forms as reported already in adult brain. However, here we found that the ramified features started to appear prominently as early as in young adult rats (1½ months of age), prior to their reproductive maturity. But conventional concept of ‘amoeboid/activated’ and ‘ramified/resting’ state of microglia has been changed now, demonstrating the functional significance of its each morphological stage in brain.

In general, the blood monocyte lineage cells mostly populate brain in late embryonic phase and disperse to colonize and gaining functional positions during neonatal phases. But in young adult onward their position and density remain similar in normal brain condition i.e., without any pathogenic interception. Their overall trend of spreading has also been described here. Therefore, the second developing window of microglial entry and settlement as mentioned previously, shows prominence in this study also. However, the overwhelming presence of dark cells during neurogenesis in early embryo as displayed by S/G staining restricted the identification of characteristic dark stained monocyte/macrophage lineage cells in that stage, and the negligible presence of CD11b+ cells in that stage fails to demonstrate the first developing window of myeloid lineage cell entry in brain. However, this observation supports the fact that in the phase of gaining adulthood there may be some possibility of entry of these cells in normal condition, reflecting a third developing window option, that remains yet controversial. Though several studies show that microglia has contribution in adult neurogenesis, but in early embryo where the brain is in the formation stage, their role is unclear in relation to neurogenesis. We found dividing undifferentiated neuroglial cells near ventricular margins, but this study could not distinguish the characteristic microglia in the early phase of embryonic brain development, in and around neurogenesis. Hence, the issue of role of microglia in early neurogenesis remains open. The present study clarifies the generalized pattern of blood borne myelo-monocytic cell dispersal and stabilization in developing brain, and hinted towards the position as well as probable mode of entry.

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