FETAL MICROGLIA AID IN DEFINING THE PROLIFERATIVE ZONES OF NEURAL STEM CELLS IN THE DEVELOPING CEREBRAL CORTEX?

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Pratiksha Atul Dighe

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Neurodevelopmental disorders, in general, and particularly autism and schizophrenia are associated with altered brain organization and structure resulting as a consequence of aberrant neurogenesis. Embryonic neurogenesis is a rapid process, resulting in the production of billions of neurons over a short period of time from the multipotent neural progenitor cells. Neurogenesis, therefore, needs to be carefully regulated to ensure the right balance between cell production and the requirements of the growing cerebral cortex. Yet, the precise mechanism by which the precursor cell production is restrained and regulated during cortical neurogenesis still remains unclear. The neural stem cells in the fetal forebrain are localized in the ventricular zone (VZ) and the subventricular zone (SVZ), the two proliferative zones where they divide to give rise to daughter neuronal cells. We have shown that microglia colonize the fetal cortex during development and regulate the number of neural and glial stem cells in the fetal brain through phagocytosis.
The goal of the study was to better understand the role of microglial cells in regulating the neural stem cell pool during neocortical development. Preliminary evidence from macaque occipital coronal sections shows that microglia are positioned as a monolayer at the interface between the VZ and SVZ. Based on this finding, we hypothesized that microglia phagocytose neural stem cells at the boundaries between the VZ and SVZ and thus, microglia may help define proliferative zones of neural stem cells by culling those cells that migrate beyond specified boundaries. To investigate this possibility, the distribution of fetal microglial cells in coronal brain sections isolated from rat (Sprague-Dawley), macaque (Macaca mulatta) and chicken (Gallus gallus domesticus) animal models at different stages of fetal brain development were analyzed. In confirmation of the preliminary results, microglia were noted to often position themselves at the border between the VZ and SVZ proliferative zones, a phenomena conserved across all the three species analyzed, thereby suggesting an evolutionary microglial patterning in the developing cortex. Moreover, the first evidence of the microglial cell line up at the interface in embryonic rat coincides with the age that reflects the peak of layer 3 neurogenesis, wherein overproduction of layer 3 neurons had been closely associated with the onset of autism like symptoms. However, with the lack of quantifiable evidence of higher proportion of microglial phagocytosis at the VZ/SVZ interface as compared to the other locations within the proliferative zones and co-labeling of microglia with markers of activated Iba1+ cells, our data remains inconclusive but definitely provides
the first proof of concept towards our hypothesis and warrants future studies aimed in this direction.

_______________________, Committee Chair
Rosalee Sprowls, Ph.D.

May 4th, 2015
Date
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. METHODS</td>
<td>9</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>14</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>31</td>
</tr>
<tr>
<td>References</td>
<td>38</td>
</tr>
<tr>
<td>Tables</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Antibody testing conditions for fixed embryonic rat brain tissue</td>
<td>30</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Microglia line up at the VZ/SVZ border in embryonic rat</td>
<td>15</td>
</tr>
<tr>
<td>2.</td>
<td>Microglia line up at VZ/SVZ border in Macaque and Chicken</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>Whole brain coronal sections of E18 rat</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>Phagocytosis of neural precursor cells</td>
<td>21</td>
</tr>
<tr>
<td>5.</td>
<td>Liposomal chlodoronate treatment of E18 embryos and analysis one day post surgery</td>
<td>23</td>
</tr>
<tr>
<td>6.</td>
<td>BV2 cultures</td>
<td>25</td>
</tr>
<tr>
<td>7.</td>
<td>Liposomal chlodoronate treatment of E18 embryos and analysis three days post surgery</td>
<td>27</td>
</tr>
</tbody>
</table>
INTRODUCTION

The cerebral cortex, the convoluted outer layer of gray matter of the cerebral hemispheres, is an important layered structure of the central nervous system (CNS). It regulates vital functions involving movement, vision, hearing, touch, learning and memory, and higher cognition. Hundreds of specialized neuronal cell types and diverse range of glial cells, constituting the bulk of the mammalian cerebral cortex, are derived from neural progenitor cells (NPCs), the neural precursor cells, through the process of neurogenesis (Florio et al., 2014; Pelvig et al., 2008). During human fetal development, over a 1000 precursor cells divide every second to produce cortical neurons (Samuelsen et al., 2003). The proliferation of the neuronal precursor cells must be carefully regulated in the developing cerebral cortex to ensure a balance between the number of neuronal cells produced to the number of cells required. The precise mechanism by which the precursor cell production is restrained and regulated, particularly during end stages of cortical neurogenesis remains unclear. If unregulated, the excess of neuronal cells generated during embryonic cortical development would result in negative consequences for brain organization and function. Aberrant neurogenesis resulting in an altered pattern of neurodevelopment, may have deleterious effects on brain development, as is commonly seen in neurodevelopmental disorders such as autism and schizophrenia (Chance et al., 2008; Wegiel et al., 2010).
Autism is characterized as a broad spectrum disorder with affected individuals exhibiting impairments in social interactions, communication and restricted, repetitive, and stereotyped patterns of behavior. According to the new data released by the Center for Disease Control and Prevention (CDC) in 2014, 1 in 68 children are reported to be autistic in the United States. Currently no known cure or effective treatment exists, but the suggested approach involves therapies and behavioral interventions looking into rectifying the symptoms associated with autism such as anxiety, depression, or obsessive-compulsive disorder. Reduction in symptoms of autism has been demonstrated in a clinical trial wherein liquid liposomal ubiquinol, diluted in milk, tea, or juice was administered to the autistic children with a view to reduce oxidative stress, an increase of which is associated with autism (Gvozdjáková et al., 2014). Schizophrenia, on the other hand, is recognized as severe and disabling brain disorder, often resulting in individuals interpreting reality abnormally. Schizophrenia affects roughly 1.1% of the U.S population and current treatment regimes include administration of antipsychotic drugs, but these tend to be associated with a number of side effects, involving the development of movement disorders (Mathews et al., 2005). Autism and schizophrenia, both display altered patterns of neurogenesis and embryonic brain development, however, the underlying cause of what triggers this aberrant pattern of neurodevelopment still remains unclear.
As mentioned above, embryonic neurogenesis is a rapid process, resulting in the production of billions of neurons over a short period of time from the multipotent neural progenitor cells. The neuronal and glial cells of the cortex are derived from the neuroepithelial cells of the neural plate and the neural tube (Merkle et al., 2006; Shioi G et al., 2008). During embryonic development, the neuroepithelial cells function as the neuronal progenitor cells (neural stem/precursor cells), undergoing self-renewal to expand their own population through symmetric division and also generate differentiated daughter cells, including post-mitotic neurons, through asymmetric divisions (Molyneaux et al., 2007; Yamashita, 2013). Neuroepithelial cells are bipolar and have one process that extends to the apical surface and a second process that extends to the basal surface of the cortex, thereby, maintaining contact with the ventricular lumen and the pial surface of the brain (Kriegstein et al., 2006; Shioi G et al., 2008). A defining characteristic of these cell types is that they undergo inter-kinetic nuclear migration which involves translocation of their nucleus within the proliferative zones of the cortex such that mitosis occurs at the ventricular surface, while S phase occurs at the basal-most region of the proliferative zone, leading to the appearance of a pseudostratified epithelium (Reiner et al., 2012).

With the onset of neurogenesis, the neuroepithelial cells, with the downregulation of certain epithelial features, give rise to radial glial cells, which retain the majority of the neuroepithelial properties including the interkinetic nuclear migration but, at the same
time, start to display characteristics of astroglial cells such as the expression of astroglial markers including astrocyte specific glutamate transporter (GLAST), Ca$^{2+}$ binding protein S100β, brain lipid-binding protein (BLBP), Tenascin C (TN-C), glial fibrillary acidic protein (GFAP), nestin and vimentin (Götz et al., 2005; Mori et al., 2005). Radial glial cells are more fate restricted compared to neuroepithelial cells, giving rise to a single cell type; either astrocytes, oligodendrocytes or neurons (Kriegstein et al., 2006; Kriegstein et al., 2009). Together, the neuroepithelial cells and the radial glial cells represent the primary neural stem cell population which are found in the ventricular zone (VZ), the first layer of proliferative zone, located adjacent to the lateral ventricle. The VZ is characterized by the presence of primary neural stem cells that express the Pax6 nuclear transcription factor, and ultimately give rise to all cells of the developing and mature CNS (Kriegstein et al., 2009).

As neurogenesis progresses, a second proliferative layer called the subventricular zone (SVZ) forms above the VZ. The SVZ is populated by the intermediate progenitor cells, a secondary neuronal stem cell population derived from radial glial cells that migrate to the SVZ during neurogenesis. The secondary neural stem cells in the SVZ express the Tbr2 nuclear transcription factor, in addition to retaining the expression of Pax6 and divide symmetrically to produce neurons (Englund et al., 2005; Kriegstein et al., 2006; Kriegstein et al., 2009). Thus, the SVZ can be viewed as a site of neurogenesis in addition to the VZ.
Microglial cells colonize the cerebral cortex during prenatal development (Verney et al., 2010) and are thought to play a central role in regulating the neuronal precursor cell pool (Cunningham et al., 2013). Microglia make up 10% of all cells of the CNS and comprise approximately 5–6% of all cortical cells (Pelvig et al., 2008; Perry and Gordon, 1988). These cells can be considered a resident macrophage-like cell type of the brain and were earlier presumed to be under a resting or static state under normal healthy conditions, till the infliction of an injury or infection, following which they get activated and take on their role as the scavenger immune defenders of the CNS (Zielasek and Hartung, 1996). However, even when in ‘resting’ state, microglia are seen to possess highly motile processes which are constantly extended and withdrawn, possibly sampling their surrounding environment as evidenced by bulbous endings, indicative of tissue material being collected. Thus, these “resting” microglia may also work effectively to control the CNS microenvironment and clear the parenchyma of accumulated metabolic products and deteriorated tissue components (Nimmerjahn et al., 2005). The microglial morphology is seen to differ throughout their life cycle, assuming various forms from active amoeboid shape to ramified cell-like appearance that is associated with the ‘resting’ state (Davis et al., 1994; Rezaie and Male, 1999). Early in embryonic stages, the CNS is majorly composed of amoeboid microglia representing an active state (Hirasawa et al., 2005). During this early embryonic period, alongside neurogenesis, the density of microglial cells is seen to rise and this continues on till nearly two weeks into the postnatal period, following which, they differentiate into ramified microglia,
characteristic of the microglial cell types found in adult CNS (Alliot et al., 1999; Ignácio et al., 2005; Xu and Ling, 1994).

As originally conceived, microglia where thought to be of mesodermal origin, as was postulated by Del Rio Hortega in 1932, based on a silver staining method and was supported by many studies in stating that the microglial cells originated from invading pial cells from the brain surface (Kaur et al., 2001). However, the origin of microglial had been a topic of debate for many years. As opposed to the notion of a mesodermal lineage, few scientists believed the microglial origin as neuro-ectodermal, possibly sharing a common progenitor with astrocytes and oligodendrocytes, which was later identified as glioblasts by Kitamura and his colleagues (Paterson et al., 1973; Fujita and Kitamura, 1975; Kitmura et al., 1984). In fact, even years later, immunohistochemical studies involving labeling microglial cells with antibodies that label neuroepithelial cells, along with in-vitro studies demonstrating the presence of microglia within cultures of late embryonic murine epidermal growth factor-derived generative zone progenitor cells supported the notion of their neuroectodermal lineage (Fedoroff et al., 1997; Papavasiliou et al., 1996). Another popular belief, which was also supported by Del Rio Hortega is that microglia are derived from the hematopoietic lineage (Hess et al., 2004; Rio-Hortega, 1939). During early embryo development, the microglia derived from circulating blood progenitors (monocytes) originating primarily within the bone marrow migrate to the neural tube, followed by extensive proliferation of these cell types within the CNS (Perry
et al., 1985). Although, the most commonly accepted theory is that microglia are derived from the hematopoetic system, a study led by Alliot et al., identified the presence of cells with a macrophage progenitor phenotype in the yolk sac before the appearance of microglial progenitors in the neural tube, thereby, suggesting brain parenchymal microglia are derived from cells originating from the yolk sac (Alliot et al., 1999). In support of this finding, studies using lectin labeling with GSA1-B4, which is specific for microglia, showed evidence of lectin labeled cells within the yolk sac of embryonic mice as early as embryonic day (E)8, confirming the derivation of microglial cells from progenitor cells present in the yolk sac (Kaur et al., 2001). Further studies confirmed on the evidence that microglia arise from erythromyeloid progenitors found within the yolk sac (Kierdorf et al., 2013).

For proper embryonic brain development and patterning, the process of neurogenesis needs have a careful balance of factors, which promote and quell neuroproliferation and migration. Such factors or even cell types, which may have an influence on embryonic neurogenesis, are not yet completely understood, but it is believed that microglial may play an important role (Noctor et al., 2004). In the developing brain, microglia are observed to phagocytize neurons and neural progenitor cells within the proliferative zones, particularly during the late stages of cortical neurogenesis, some which even include stressed yet viable neurons along with the dead and dying neurons (Brown et al., 2014; Cunningham et al., 2013). Eliminating or
activating microglia in the fetal brain has direct consequences on neuronal proliferation and migration (Breunig et al., 2011; Cunningham et al., 2013). Therefore, microglial cells can be considered as an important cell population in the fetal neocortex that regulate the size of the neuronal stem cell pool.

Our preliminary evidence suggests that microglia are often positioned along the boundary that separates the VZ and SVZ proliferative zones (Cunningham et al., 2013). If proven that microglia may preferentially phagocytose more neural stem cells at the VZ/SVZ boundary as compared to other locations in or near the proliferative zones, we can hypothesize that that fetal microglia may function to define the boundaries of neural proliferative zones in the developing brain by culling those neural stem cells that migrate beyond specified VZ and SVZ boundaries. With the ultimate goal to better understand the role of microglial cells in regulating the neural stem cell pool during neocortical development, the focus of the project was to investigate this hypothesis by quantifying the distribution of fetal microglial cells in the developing fetal brain, and comparing the apparent function of microglia positioned at the boundary of the proliferative zones versus those positioned within proliferative zones. Selective depletion of microglial cells in the developing cortex was attempted in order to analyze its effects on neural stem cell pool and their distribution within the proliferative zones. The involvement of microglia in shaping the brain cytoarchitecture and understanding the way in which they interact with the neural precursor cells would give an insight into the mechanisms involved in regulating neurogenesis and in identifying potential targets for therapeutic intervention in the attempt to treat neurodevelopmental disorders.
METHODS

Animals

All animal procedures on timed pregnant Sprague Dawley rats were approved by The Institutional Animal Care and Use Committee of the University of California, Davis and were conducted in accordance with the National Institutes of Health guidelines for the use of animals in research (Protocol 16368). Sections of fixed macaque (*Macaca mulatta*) brain tissue were a generous gift from the laboratory of Dr. David Amaral. Procedures on macaques were approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Protocol 12139) and strictly adhered to National Institutes of Health policies on primate animal subjects. Rat brains used included E15, E17, E18, E19, E20 and E21. Macaque tissue was obtained from animals at E50, E65, and E80. Pre-fixed, pre-stained tissues of embryonic chicken coronal brain sections prepared at the Noctor Lab were used for analysis.

Embryonic perfusions

For fixed slices, embryonic rats of either sex were removed from the dam and were transcardially perfused with 4% PFA. The dams were anesthetized using inhalant isoflurane delivered through an Isoflurane Vaporizer. First, the dam was placed in an induction chamber with an inflow of 5% isoflurane, followed by placing her on a warming pad with isoflurane (reduced down to 3%) administered by nose cone continuously. After ensuring anesthetic induction through lack of tactile stimulus, a midline incision through the skin and abdominal lining was made approximately 2 inches from base of tail dorsally. The embryos were removed individually, beginning at the top
of the uterine horn. Embryos were anesthetized by placing them on ice and then were pinned down onto a dissecting dish while being visualized through a dissecting microscope. With the aid of microscissors, a lateral incision was made through the abdominal wall just below the ribcage, after which a small incision was made in the diaphragm and continued on till along the entire length of the rib cage. The sternum was lifted away, exposing the heart. The right atrium is snipped with the aid of scissors and a needle is injected into the left ventricle. Approximately 5-8 ml of PBS was flushed through the embryo transcardially, followed by 5-8mls of cold 4% paraformaldehyde over the course of 5 minutes with constant pressure. Brains were removed and processed (Martinez-Cerdeno et al., 2012), post-fixed in 4% PFA overnight and transferred 30% sucrose + 0.1% sodium azide, stored at 4°C until required. For preparing coronal sections, the brains stored in sucrose were washed 3 times with PBS, frozen in optimum cutting temperature formulation (O.C.T) cryomolds, sectioned coronally on a cryostat, mounted on Superfrost Plus slides (Fisher) and stored at −20°C until further use (Martinez-Cerdeno et al., 2012).

In utero intracerebral injections: selective depletion of microglia

Liposomes containing PBS or clodronate (ClodronateLiposomes.com, The Netherlands) were prepared as described previously (Van Rooijen et al., 1996). The refrigerated liposomes were warmed and brought to room temperature prior to the start of the surgery. Timed pregnant rats were anesthetized at E18 or E20. 2–3 µl (stock concentration: 5mg/ml) of clodronate or PBS liposomes were injected into the lateral ventricles of the embryos using glass micropipettes. Prior to use, the liposomal solutions
were vortexed gently in order to ensure uniform suspension and mixed with blue dye to enable visual aid while injecting into the lateral ventricles. Few embryos were left untreated to serve as control. The uterus was replaced, the incision sutured, and the pregnant animal allowed to recover. One to three days post injection, embryos at E19, E21 or pups at P1 were transcardially perfused with 4% paraformaldehyde, coronally sectioned and immunostained.

Immunohistochemistry (IHC)

Slide mounted brain tissue sections were prepared as described above. The slides were warmed to room temperature and the slices on the slide were circled with a PAP pen to form hydrophobic barrier well and. The slides were washed with 0.1M PBS, 3 times and then subjected to antigen retrieval by boiling sections, using a microwave, or steaming, using a steam cooker, in 10 mM Citrate Buffer (pH 6.0) containing 10 mM Citric Acid and (v/v) 0.5% Tween-20 for fifteen minutes. This was followed by a blocking step, incubating the sections with 10% donkey buffer (100-200 ul per slide) containing (v/v) 10% fetal donkey serum, 0.1% Triton X-100, and (w/v) 0.2% gelatin for one hour at room temperature (RT). Sections were then subjected to incubation with primary antibody buffer containing the primary antibodies (as listed below) along with (v/v) 2% fetal donkey serum, 0.02% Triton X-100, and (w/v) 0.04% gelatin. Incubation times with the primary antibodies ranged from 12-72 hr at room temperature or 4°C (refer table below). Post incubation with primary anitbodies, sections were again rinsed in 0.1M PBS followed by incubation for 1.5 hr at RT in secondary antibody buffer containing secondary antibodies (as listed below), (v/v) 2% fetal donkey serum, 0.02%
Triton X-100, (w/v) 0.04% gelatin, and DAPI 1:500. Sections were rinsed again in 0.1M PBS and coverslipped with Mowiol. For certain antibodies, the antigen retrieval step was excluded to determine if the antigen retrieval method damaged antibody binding. Moreover, Triton X-100 was not added as a part of the donkey buffer (the 10% used for blocking and the 2% used for antibody solution preparations), again to test for differences in antibody binding. Primary antibodies: mouse anti iNOS (1:30 – 1:100, stock conc: 0.5mg/ml) (R&D Systems), mouse anti CD68 (1:20 – 1:500, stock conc: 30mg/L) (Dako), goat anti Iba1 (1:200, stock conc: 0.5mg/ml) (Abcam), rabbit anti HLA-DR (1:50 – 1:100, Bios), mouse anti CD11b (1:20 – 1:100, stock conc: 1mg/ml) (Abcam), rat anti CD11b (1:50 – 1:100, stock conc: 1mg/ml) (Abcam), rat anti F4/80 (1:30 – 1:100, stock conc: 0.5mg/ml) (Ebioscience), mouse anti- Pax6 (1:50-1:100, stock conc: 1mg/ml) (Abcam), rabbit anti- Pax6 (1:100, stock conc: 2mg/ml) (Covance) and chicken anti Tbr2 (1:500, stock conc: 0.5mg/ml) (Millipore). Secondary antibodies (1:200, stock conc: 1.5mg/ml) (Jackson Immunoresearch): Donkey anti-mouse, donkey anti-rabbit, donkey anti-goat and donkey anti-rat. Secondary antibodies were conjugated to Dylight 405, Cy2/Dylight 488 (or Alexa Fluor 488), Cy3/Dylight 549 (or Alexa Fluor 546), or Cy5/Dylight 649 (or Alexa Fluor 647).

For the organotypic slice cultures, the slices were allowed to warm up to room temperature and transferred to new six well plates with net wells and 0.1M PBS. This was followed by the similar washing, blocking and incubation with primary and secondary antibodies as described above. The immunostained slices were kept stored in 4°C, submersed in 0.1M PBS, until viewed and imaged under the confocal microscope.
**BV2 cells**

BV2 cells, derived from primary mouse microglia cells, served as a suitable model for *in vitro* studies on microglia (Bocchini *et al.*, 1992). In order to determine the efficacy of the liposomes containing clodronate in depleting microglia, we sought to assess the extent of microglial clearout in the presence of clodronate *in vitro*. The cells were grown in 6-well plates and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), until they were fairly confluent (65-70%). BV2 cells were treated with liposomes with clodronate (1:5 and 1:25), liposomes with PBS (1:5 and 1:25) or as control, for up to 72 hrs. Cells were checked visually under phase contrast microscope for reduction in cell numbers (0, 12, 24, 48 and 72 hours). Three days post treatment, cells were fixed with 4% PFA and were evaluated for protein expression of Iba1, Tbr2 and Pax6 using immunocytochemistry. Antibodies evaluated were used at same concentrations as IHC.

**Imaging**

Imaging of the immunostained slides/organotypic slices were performed using an Olympus FV1000 confocal microscope. Z-stack images were acquired using the confocal microscope to view the tissue sections in 3D plane. Microglia & phagocytic events were be localized to anatomical regions defined based on cytoarchitectonics using the nuclear stain DAPI. Excitation/emission wavelengths were 405nm (DAPI), 488nm (Cy2), 568/590nm (Cy3) and 685/690nm (Cy5). Images were contrast-enhanced, assembled and false color added using Adobe Photoshop (Noctor *et al.*, 2004).
RESULTS

Microglia line up at the VZ/SVZ border

The regional distribution of microglia was analyzed in the cortex of prenatal rats, spanning from E15 – E20 using antibodies against ionized calcium binding adapter molecule 1 (Iba1), which is specific in labeling microglia (Imai et al., 1996). VZ and SVZ borders were defined by labeling with antibodies against nuclear transcription factors, Pax6 and Tbr2 respectively. It has been previously noted that Iba1+ cells are seen as a monolayer at the interface of the VZ and SVZ border in macaque tissue and this phenomena is observed as early as E50 (Fig. 2e), even though Iba1+ cells sparsely populate the cerebral cortex at this stage in primates (Cunningham et al, 2013). At E18, during the peak of layer 3 neurogenesis in rat (Bayer and Altman, 1991), we noted a similar pattern of microglial cell line up at the VZ/SVZ border (Fig. 1a). This preferential positioning of Iba1+ cells is seen to be present consistently till E20 (Fig. 1b-c), near the end of cortical neurogenesis (Bayer and Altman, 1991). However, at E17, the distribution of Iba1+ cells was quite sparse and neither could we note any of these cells at the VZ/SVZ interface (Fig. 1d). A similar trend of the presence of microglia forming a band at the VZ/SVZ interface was noted in the chicken neocortex (Fig. 2a-c), with the earliest evidence seen at E7 (Fig. 2c). For chicken coronal sections, the microglial cells were identified with antibodies against tomato lectin.
Figure 1. Microglia line up at the VZ/SVZ border in embryonic rat. a–c, Monolayer of Iba1+ microglial cells (green) is seen interface of the VZ and SVZ (white arrow). d, Microglia sparsely colonize the neocortex at E17. Line up microglia at VZ/SVZ border is absent at this age. LV - lateral ventricle. Image magnification: a-c = 10X, d = 20X.
Figure 2. Microglia line up at VZ/SVZ border in Macaque and Chicken. a-c, Monolayer of tomato lectin positive microglial cells (green) is seen at the border between VZ and SVZ (white arrow) in chicken neocortex. Earliest evidence is seen at E7 (c). d-e, Iba1+ microglia at the VZ/SVZ interface in macaque tissue. V = Ventricle. Image magnification: a-b = 10X, c-e = 40X

Whole brain coronal section of E18 rat

In order to identify the exact location within the cortex where the microglia align at the border between the two proliferative zones and E18 being identified as the age at which this phenomena was first seen to occur, whole brain coronal sections of E18 rat, starting from the rostral anterior end to the caudal posterior spanning the occipital, parietal and frontal lobes, were analyzed. Iba1+ microglia were observed to line up
between the Pax6-Tbr2 bands of the proliferative zones in all the coronal sections (Fig. 3), particularly in the cell dense proliferative regions of the dorsal and dorsolateral cortex that surrounds the lateral ventricle during cortical development and visualized more prominently in sections towards the posterior end of the brain (Fig 3c, d).
Figure 3. Whole brain coronal sections of E18 rat. Iba1+ microglial cells (green) are seen lining
up at the interface between the VZ (blue) and SVZ (red). a, e, Coronal section from the anterior most end of the frontal lobe. b, f, Coronal section from the posterior most end of the occipital lobe c, Mid brain coronal section with microglia line up at the VZ/SVZ border (white arrows) at the dorsal and dorsolateral cortex, (g) the hippocampal region and basal telencephanic plate (inset). d, Posterior coronal section with microglia positioned at the VZ/SVZ border (white arrows) at the dorsal, (h) dorsolateral, ventral cortex and the hippocampal region. Image magnification: a-d = 10X, e-h = 40X. cx = cortical neuroepithelium, hi = hippocampus, bta = basal telecephalic plate, DCx = Dorsal cortex, DlCx = Dorsolateral cortex, VCx = Ventral cortex, LV = Lateral ventricle, VZ = ventricular zone, SVZ = sub ventricular zone.

Interestingly, a monolayer of Iba1+ microglia was noted even in the developing hippocampal region (Fig. 3c, d) as well as the basal telencephanic plate (Fig. 3c inset), which surrounds the lateral ventricle.

The morphology of the Iba1+ microglia at the VZ/SVZ interface in the cortex of the embryonic rats at E18 were often observed to be rounded ‘amoeboid’ (Fig. 3h), typical characteristic of activated microglia (Rezaie and Male, 1999), while some even exhibited ramified morphology, representing cells that appear to be resting microglia. Mainly the monolayer of microglial cells at the VZ/SVZ border at the hippocampus were the ones to exhibit morphologies characteristic of resting microglia (Fig. 3g).

**Phagocytosis of neural precursor cells**

Microglia are known to colonize the different layers of the cerebral cortex at various stages of development with a higher proportion of these cells being observed in the proliferative zones (Verney et al, 2010). Consistent with this view, our previous studies have demonstrated an increase in number of microglia in the cerebral cortex at each stage of development and when looked at the macaque SVZ, microglia were noted to engulf
and phagocytose Tbr2+ and Pax6+ cells in the SVZ at all stages of development (Cunningham et al., 2013). We therefore examined the relationship between the microglia and neural precursor cells at the stage of cortical development when the microglia first appear to line up between the VZ and the SVZ border and study the phagocytic events at this boundary. Few events of what seemed like microglial phagocytosis of Pax6+ cells were recorded at the VZ/SVZ interface. Pax6+ nuclei were located within the somata of Iba1+ microglia (Fig. 4a-d), while in most events we could observe the microglial processes reaching out and surrounding the precursor cells (Fig. 4e-h), possibly with the next stage being the complete engulfment and degradation of the precursor cells. However, we also noted a few events of Tbr2+, Pax6+ precursor cell phagocytosis of microglia in the SVZ (Fig. 4i-l), as has been noted previously. Further studies with time lapse imaging by way of in utero intracerebral electroporations in E18 rat embryos, with a reporter gene expressing plasmid that labels the neural precursor cells, would need to be carried out to quantify the number microglial phagocytic events at the VZ/SVZ border in comparison to the proliferative zones.
Figure 4. Phagocytosis of neural precursor cells. 

- **a – h**: Iba1+ microglia (green) at the VZ/SVZ interface in the E18 rat cortex contact and envelope Pax6+ (red) neural precursor cells. 

- **d, h**: Nuclei stained with DAPI. 

- **f-h**: Iba1+ microglia cell process (green) surrounds a pax6+ precursor (red). 

- **i-h**: Iba1+ microglia (green) in the SVZ contacts and starts to engulf Tbr2+ (red) neural precursor cell. 

Image Magnification: 

- **a, e, i = 10X** 

- **b-d, f-h, j-l = 40X**
Eliminating microglia using liposomal clodronate

In trying to study the role of microglia in defining the proliferative zones in the developing cortex and its effect on the neural precursor cell pool, the aim of the next set of experiments was to examine the changes in the neural precursor cell distribution and pool size in the VZ and SVZ zones when microglia are eliminated from the cortex. Liposomal clodronate is an FDA approved drug used specifically for the treatment of human bone conditions (Valimaki et al., 2002) and is known to selectively kill macrophages and microglia (Kumamaru et al., 2012, Van Rooijen and Sanders, 1994). Previous studies at our lab, in treating organotypic cultures as well in utero intracerebral injections of liposomal clodronate into the lateral ventricle of E16 and E20 embryos had resulted in dramatic decrease in the number of microglial cells with subsequent increase in the number of Pax6+ and Tbr2+ cells (Cunningham et al., 2013). Therefore, with E18 identified as the age of the onset of the microglial monolayer patterning between the VZ and the SVZ border, liposomal clodronate was injected (2-3µl for each embryo, conc: 5mg/ml) intracerebrally in utero into the lateral ventricles of E18 rat embryos. For the first set of experiments, animals were sacrificed one day later (E19) and were sectioned to immunostain for with antibodies to label neural precursor cells (Pax6, Tbr2) and microglia (Iba1). Unfortunately, only a 11% decrease (Fig. 5b, g) in the number of Iba1+ microglia cells in embryos treated with clodronate was observed on quantifying the cells in the E19 dorsolateral neocortex in 200 µm wide bins. However, on closer look at the morphology of the Iba1+ microglia for the clodronate treated embryos, they appear to be morphologically distorted and may be undergoing the process of degradation (Fig. 5e) as
compared to the control (Fig. 5d) and liposomal PBS treated embryos (Fig. 5f) which still maintain active amoeboid microglial morphology.

**Iba1/DAPI**

- E19
- CL
- PBS

**Iba1/DAPI**

- E19
- CL
- PBS

**E19**

![Graph showing number of cells per 200um wide bins](image)

Figure 5. Liposomal clodronate treatment of E18 embryos and analysis one day post surgery.
E18 embryos injected with liposomal clodronate, liposomal PBS and untreated controls visualized one day post injections. a, A coronal section of E19 rat brain stained for microglia (green) and nuclei (DAPI, blue). Microglia populate the cortex. b, The number of microglia are hardly reduced after in utero intraventricular injections of liposomal clodronate at E18. c, Placebo treated coronal section of E19 rat. g, Histogram showing that one day after E18 in utero intraventricular injection of liposomal clodronate, there is no significant decrease in the number of microglia. The Iba1+ cells of clodronate treated embryos (e) appear to lose their amoeboid morphology when compared to the untreated (d) and placebo treated (f) controls. Error bars show standard error (n = 3). LV = lateral ventricle, CNTRL = control, CL = clodronate, PBS = phosphate buffered saline. Image magnification: a-c = 10X, d-f = 40X. Scale: a-f = 100µm

Since the *in utero* injections at E18 and analyzing one-day post surgery did not work to eliminate the microglia as effectively as what has been witnessed at our lab before, an in-vitro assay with BV-2 cell cultures and their elimination under the influence of clodronate was performed in order to test for the efficacy of our batch of clodronate liposomes. BV2 cells are a microglial primary mouse microglia cells and ideally when incubated with media containing clodronate, we should be able to see a clear wipe out of the cells from culture, as is witnessed with the MG-5 mouse microglial cell line (Kumamaru et al., 2012). No observable differences in the number of BV2 cells under the different conditions of treatment, namely with clodronate, placebo and control, were noted when looked at visually under the phase contrast microscope 24 hr post treatment. It was not until the end of 48 hrs post treatment that a reduction of the number of BV-2 cells adhered to the culture plate and an increase in the number of floating cells was noted, indicating onset of microglial elimination in cultures treated with clodronate.
Three days after treatment (72hrs), BV-2 cell cultures were fixed and immunostained with antibodies against Iba1 to label the microglia. A dramatic 90% decrease in the number of Iba1+ BV-2 microglial cells was observed for cultures treated with clodronate at 1:5 dilution when compared to control. Again, a 75% reduction in microglial cell numbers was noted for cultures treated with clodronate at 1:25 dilution, indicating a dose dependent effect of clodronate in eliminating the microglia. No significant differences were observed when comparing placebo to control.
Figure 6. BV2 cultures. 

- a, c, Liposomal clodronate treated BV-2 cells exhibit reduction in number of Lba1+ cells (green) in culture after three days of treatment. 
- b, d, BV2 cell cultures treated with placebo. 
- e, Histogram showing that three days after in vitro treatment of BV2 microglia cells with liposomal clodronate, there is significant decrease in the number of microglia cells. Error bars show standard error. T-test, double black asterisk: p<0.01. CNTRL = control, CL = clodronate, PBS = phosphate buffered saline. Image magnification: 40X. Scale: a-d = 100µm.

Based on the results of the BV2 cell cultures with clodronate, E18 embryos were injected in utero with liposomal clodronate, similar to the previous test set, but were
examined three days post treatment. At E21, three days after E18 in utero intracerebral injections of liposomal clodronate, a bare 30% reduction in the number of Iba1+ microglia were noted in the developing cerebral cortex (Fig. 7b, d-e).

**Iba1/Pax6**

![Image of Iba1/Pax6](image)

**Iba1/DAPI**

![Image of Iba1/DAPI](image)
Figure 7. Liposomal chlodronate treatment of E18 embryos and analysis three days post surgery.

E18 embryos injected with liposomal chlodronate, liposomal PBS and untreated controls visualized three days post injections. a, c, A coronal section of E21 rat brain stained for microglia (green) b, d, Only a slight reduction in the total number of Iba1+ microglia after in utero intraventricular injections of liposomal clodronate at E18 analyzed three days post treatment. g, Histogram showing that three days after E18 in utero intraventricular injection of liposomal clodronate, there is no significant decrease in the number of microglia cells. Error bars show standard error. LV = lateral ventricle, CNTRL = control, CL = clodronate. Image magnification: a, b = 10X, c, d = 40X. Scale: a-d = 100µm.

With the earlier success in eliminating nearly 90% of the microglial cells in vivo in our previous studies, wherein the lateral ventricle of E20 embryos were injected with liposomal clodronate and sacrificed three days later to assess the relationship between microglia and neural precursor cell pool (Cunningham et al, 2013), a repeat of this particular experiment was performed with injecting E20 embryos, rather than E18, with the aim to assess the level of microglial depletion after three days of treatment with
clodronate. However, out of the three litters that were injected at E20, all were aborted by the dam by the second day post the in-utero surgery.

*Antibody testing conditions for microglial cell activity at VZ/SVZ border*

After having determined E18 as the age at which microglia are positioned at the boundary between the VZ and SVZ and their specific locations within the developing brain, further steps were to identify the activation states and the specific roles played during neurogenesis by the identified microglial cells. For this purpose, co-labelling with antibodies against typical markers of microglia such as F4/80, iNOS, CD68, HLA-DR and CD11b. Multiple concentrations of the antibodies were tested, ranging in concentrations from 1:20 to 1:500 (considering the manufacturer’s recommended concentrations for use with fixed tissue). Different IHC conditions were tested, involving eliminating the antigen retrieval step, lowering or eliminating TritonX from the donkey buffer used for blocking and in preparation of the primary/secondary antibody solutions, alternating the incubation time and temperatures, etc as listed below in Table 1. No positive labeling of the fixed embryonic rat brain tissues were noted but for the exception of one incident where co-labeling of microglial cells with iNOS was observed with an E18 coronal section which performing the standard IHC protocol (refer methods) with antigen retrieval, incubation with primary mouse anti-iNOS antibody for 72 hr at 4°C and secondary antibody incubation at room temperature for 2 hrs. However, with repeat experiments employing the same conditions, no positive staining was visualized and only a high background was observed.
Table 1. Antibody testing conditions for fixed embryonic rat brain tissue. Neg = negative, RT = room temperature.

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<th>Antibody Testing Conditions</th>
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<td>IHC standard protocol</td>
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<td></td>
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<td>Donkey buffer without Triton X (10% blocking, 2% incubation buffer)</td>
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<td>all conc. tested = neg</td>
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Table 1. Antibody testing conditions for fixed embryonic rat brain tissue. Neg = negative, RT = room temperature.
DISCUSSION

Aberrant neurogenesis, altering brain organization and structure, is often associated with neurodevelopmental disorders such as autism and schizophrenia. Therefore, a careful understanding of the mechanisms that regulate neurogenesis as well as the roles played by cells and additional factors that support neurogenesis is critical when considering development of therapeutic interventions to treat or even prevent the onset of neurodevelopment diseases. The involvement of microglia in neural development and thus, brain structure and organization has been implicated by many studies. For example, exogenous addition of microglia or media conditioned by microglia to culture of basal forebrain progenitor cell resulted them in differentiating into cholinergic neurons (Jonakait et al., 1996). Similarly, soluble factors released by microglia direct the migration and differentiation of neural precursor cells (Aarum et al., 2003), thereby suggesting the microglial cell involvement in neurogenesis.

It has been long recognized that regressive events are involved when it comes to neurogenesis (Cowan et al., 1984) and studies at our lab provide evidence pointing towards a possibility that microglia play a role in reducing the size of the precursor cell pool as they were shown to phagocytose viable precursor cells in the neural proliferative zones (Cunningham et al., 2013). Taking this data into consideration, there is a definite possibility that a surplus of precursor cells are generated during cortical development and excess cells are culled by microglia. The location and pattern of positioning of microglia across the cortex may have implications in determining the development of cytoarchitectural and functional differences witnessed across cortical areas. With
preliminary evidence showing that microglia are often positioned along the boundary that separates the VZ and SVZ proliferative zones, we could hypothesize that fetal microglia define the proliferative zones of the neural stem cells by culling those cells that migrate beyond the specified VZ and SVZ boundaries. In support of our preliminary evidence, we noted that microglia position themselves between the VZ/SVZ interface, early in embryonic cortical development, particularly as early as E18 in rat (Fig. 1a), E50 in macaque (Fig. 2e) and E7 in chicken (Fig 2b, c). This preferential microglial positioning at the VZ/SVZ border is thus, seen to be evolutionarily conserved across species, further supporting our hypothesis in the possible role of microglia in defining the proliferative zones of the neural stem cells.

As time sensitive developmental periods exist for structure and function in different brain regions, factors such as location and timing are critical in affecting the developmental route. For example, a single factor, such as an infection or insult, may result in a variety of cortical disorders and depending on the prenatal timing of the insult, can affect cells in one layer while sparing them in another. In view of this, we noted that the earliest onset of the microglial line up between the boundaries of the proliferative zones in embryonic rat was noted at E18, an age reflecting the peak of layer 3 neurogenesis. Overproduction of neurons in layer 3 is associated with neocortical malformation and malfunction along with association of autism like behaviors (Fang et al., 2014). Although we noted a conservation of microglial positioning at the VZ/SVZ border in the cortex across species, the timing of this onset at the time of layer 3 neurogenesis will have to be confirmed, as we noted the microglial line up at E50 in
macaque, when cortical layer 6 neurons are generated (Rakic, 1974) and E7 in chick and coronal sections from both these species at earlier embryonic ages were not looked at.

When examining E18 whole brain coronal sections in order to map the regions of the developing brain where the microglia position themselves at the boundary between the two proliferative zones, the Iba1+ microglia appeared as a monolayer between the two zones throughout the embryonic brain, starting from coronal sections taken from the anterior most lobe, right till coronal sections taken from the posterior end of the brain. More specifically they were noted to line up at the VZ/SVZ border at the dorsal and dorsolateral cortical areas surrounding the lateral ventricle, as well as in the hippocampal region and in the basal telencephalic plate of the coronal sections taken towards the posterior end of the brain. Given this finding and with further positive proof of microglia aiding in determining the borders of the proliferative zones, we may be able to gain a better understating as to why we note cytoarchitectural changes and increase in neuronal cell densities in the cortex of children suffering from neurodevelopmental diseases (Courchesne et al., 2011, Oblack et al., 2011).

Microglial phagocytosis of neural precursor cells was noted for the Iba1+ cells lined up at the VZ/SVZ interface. Most events in the fixed E18 brain tissue demonstrated possible contact and engulfment of Pax6+ cells of VZ by the microglial cells that may be migrating to the SVZ (Fig. 4d, h). Moreover, the microglia at this position more often tend to exhibit an amoeboid morphology, indicating their high active behavior when present at the boundary. This data provides us with proof of concept of our hypothesis,
however, events of microglial phagocytosis of neural precursor cells were also recorded in the SVZ (Fig. 4l) and VZ (data not shown). Therefore, in order to quantify if the microglial phagocytosis occurs more frequently at the boundary between VZ and SVZ, compared to other locations within the proliferative zones, further studies with time lapse imaging, involving the use of specific reporter gene plasmids to label the neural precursors and isolectin antibodies to label microglia, the interaction and activity of microglia can be traced in real time over a specific period.

With the hope to study the changes in neuronal precursor cell numbers and cytoarchitecture of the VZ and SVZ in the absence of microglia, *in vivo* and *in vitro* experiments involving the treatment with liposomal clodronate were performed. Clodronate is a bisphosphonate which is widely used to treat osteolytic diseases and a known inducer of macrophagic and microglial apoptosis. Since free clodronate is highly hydrophilic, it may face issues in crossing the cellular phospholipid membranes (Russell *et al.*, 1999). Therefore, liposome encapsulated clodronate was developed and has been successfully used for macrophage and microglial elimination *in vivo* (Kumamaru *et al.*, 2012, VanRooijen *et al.*, 1996). The liposomes are immediately recognized as foreign particles by phagocytic cells, namely macrophages and macrophage like microglial cells, which engulf the liposomal clodronate. Post phagocytosis, the various enzymatic activity of the phagolysosomes results in releasing the clodronate inside the cell, followed by a cascade of events, ultimately leading to the apoptosis of the phagocytic cell that internalized the clodronate (VanRooijen *et al.*, 1996). Unfortunately in the set of *in vivo* experiments performed here with *in utero* intracerebral injections of liposomal clodronate
into the lateral ventricles of E18 embryonic rats, spanning one to three day treatment regimes, did not result in eliminating microglia from the cerebral cortex. However, it must be noted that previous experiments at our lab, involving injecting liposomal clodronate at E16 and E20 resulted in nearly 90% reduction in the number of microglial cells when analyzed one to three days post treatment (Cunningham et al, 2013). When assayed for its effectiveness in-vitro using BV2 microglia cell line, we expected to see some amount of reduction in the number of microglia after 24 hr of treatment and almost total elimination at the end of three days, as has been reported with MG-5 microglial cell lines in-vitro when exposed to liposomal clodronate (Kumamaru et al., 2012). In contrast, no differences in microglial cell number was noted 24 hrs post treatment (data not shown) and even after 72 hrs of exposure, although a significant reduction in the number of BV2 microglial cells was observed, it did not result in a clear wipe out, even after treating with a high concentration of liposomal clodronate (1:5 dilution). Future studies with respect to this experiment may involve testing with a new batch of clodronate as well as comparing against clodronate liposomes acquired from a different manufacturer, such as the one used in our published study by Cunningham et al in 2013.

Investigations into the possible activity and characteristics displayed by microglial cells that line up at the border between the VZ and the SVZ proliferative zones would give us further insight into their functional roles during cortical neurogenesis under normal physiological conditions. For this purpose, we tried to analyze for co-labeling of microglial cells with few well-characterized markers generally expressed by active microglia which included iNOS, CD68, CD11b, F4/80 and HLA-DR. Inducible
nitric oxide synthase (iNOS), responsible for the production of Nitric oxide (NO), is largely expressed by microglia during normal development (Sierra et al., 2014) and localized production of nitric oxide is required for the vertebrate brain to develop normally (Peunova et al., 2001). F4/80, a part of the EGF-TM7 protein family and HLA-DR, class II major histocompatibility complex, are both extremely referenced macrophage markers, known to be involved in the process of antigen presentation and are constitutively expressed by microglial cells (Benvenisten et al., 2001, Carson et al., 1998, Gehrmann et al., 1993). CD68, a transmembrane glycoprotein and CD11b, a surface molecule belonging to the integrin family, are implicated to be expressed on the surface of macrophages, with possible roles in the process of phagocytosis (Perego et al., 2011).

With a series of immunohistochemistry staining tests along with varying the IHC staining protocol (refer details in Table 1) and testing with fixed frozen tissue from different embryonic ages (E18-E21), no positive results were noted, although previous studies at our lab have demonstrated co-labeling with most of the markers mentioned here by the microglia in the proliferative zones of the developing neocortex (Cunningham et al., 2013). Incorporating biotin labeling in the IHC protocol can be employed in future testing to amplify the signal, without confounding fluorophore overlap. Alternatively, antibodies raised in different species or from alternate manufacturers at varying concentrations could also be tested.

In conclusion, although the data presented here does not substantially support the proposed hypothesis of fetal microglia cells acting as an aid in defining the proliferative zones of the neural stem cells in the developing cerebral cortex, it does clearly point out
to an evolutionarily conserved behavior of microglia positioning themselves at the border that separates the two proliferative zones throughout the cerebral cortex and thus, warrants the need of further investigations as have been proposed above.
REFERENCES


Gehrmann J, Banati RB,


