PERIPHERAL LIPOPOLYSACCHARIDE CHALLENGE TRANSIENTLY REDUCES HIPPOCAMPAL NEUROGENESIS AND ACTIVATES MICROGLIA

By

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To my family and friends

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
CA	Cornu ammon
COX-1	Cyclo-oxygenase-1
APCs	Antigen-Presenting Cells
BBB	Brain blood barrier
CNS	Central nervous system
CRISP	Computing robotics and imaging for surgery platform
I-LPS	Iodine lipopolysaccharide
IL-1β	Iinterleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
INF- γ	Interferon-γ
LPS	Lipopolysaccharide
NSAID	Non-steroidal anti-inflammatory drug
SGZ	Subgranular zone
SVZ	Subventricular zone
NGF	Neural growth factor
NPC	Neural progenitor cells
TLR4	Tool-like receptor
TNF-α	Tumor necrosis factor α
TNF-RI	Tumor necrosis factor Receptor I
TNF-RII	Tumor necrosis factor Receptor II

Abstract of Thesis Presented to the Graduate School
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By

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Neuroinflammation, through microglial activation, significantly compromises adult hippocampal neurogenesis in the days after its administration and impairs memory weeks later. We injected adult female mice intraperitoneally with saline or bacterial lipopolysaccharide (LPS) to induce an inflammatory and then neuroinflammatory response and quantified microglial activation in the days (5, 24, 48 and 96h) and weeks (1 and 4 weeks) later. We also tested whether neurogenesis was compromised weeks after LPS injection by injection the DNA synthesis marker bromodeoxyuridine (BrdU) either 5h, 1, 2, 3 or 4 weeks after LPS and then perfusing the mice one week after BrdU. IBA-1^+ microglia became hypertrophic within 24h and expressed higher levels of the activation marker CD11β (but not the phagocytic marker CD68) within 4 days of LPS injection. These markers of activation were not elevated in IBA-1^+ cells weeks after LPS injection. Consistent with previous work, we detected lower levels of neurogenesis in LPS- versus saline-treated mice in the week after LPS, but did not find long-term effects of LPS on neurogenesis. Our results suggest that neurogenesis only requires protection against infection-induced neuroinflammation in the week after infection. However, the

neuroinflammation that accompanies injury or disease may require more prolonged modulation to facilitate neural engineering strategies seeking to replace neurons.

CHAPTER 1
INTRODUCTION

Adult Neurogenesis

Neurogenesis in humans and all other mammals studied occurs in the olfactory bulb and hippocampal dentate gyrus throughout life (see Figure 1-1; Altman & Das 1965, Lois & Alvarez-Buylla 1994, Cameron *et al.* 1993). Neural progenitor cells (NPCs) resident to the subventricular zone (SVZ) chain migrate several cm through the rostral migratory stream before fully differentiating into dopaminergic neurons of the periglomerular layer and GABAergic neurons of the granule cell layer in the olfactory bulbs (Lois & Alvarez-Buylla 1994). These new neurons are thought to integrate functionally into the neural circuits that mediate olfaction because reduced olfactory neurogenesis is associated with reduced olfactory discrimination scores (Magavi *et al.* 2005). NPCs resident to the subgranular zone (SGZ; between the hilus and the granule cell layer) of the hippocampal dentate gyrus migrate locally a little deeper into the granule cell layer before fully differentiating into glutamatergic granule neurons (Palmer *et al.* 1999, Cameron et al. 1993). New neurons are thought to integrate functionally into the neural circuits that mediate hippocampus-dependent behaviors, such as spatial navigation and the processing of contextual information because new neuron number generally relates to learning and memory scores in hippocampus-dependent tasks (Squire 1992, Aimone *et al.* 2011, Deng *et al.* 2010). While neurons are only produced in significant numbers postnatally in the hippocampal dentate gyrus and olfactory bulbs, NPCs harvested from any brain region can produce neurons and glia in the appropriate culture conditions, suggesting that the whole brain is capable of regeneration (Roy *et al.* 2000, Palmer *et al.* 2001).

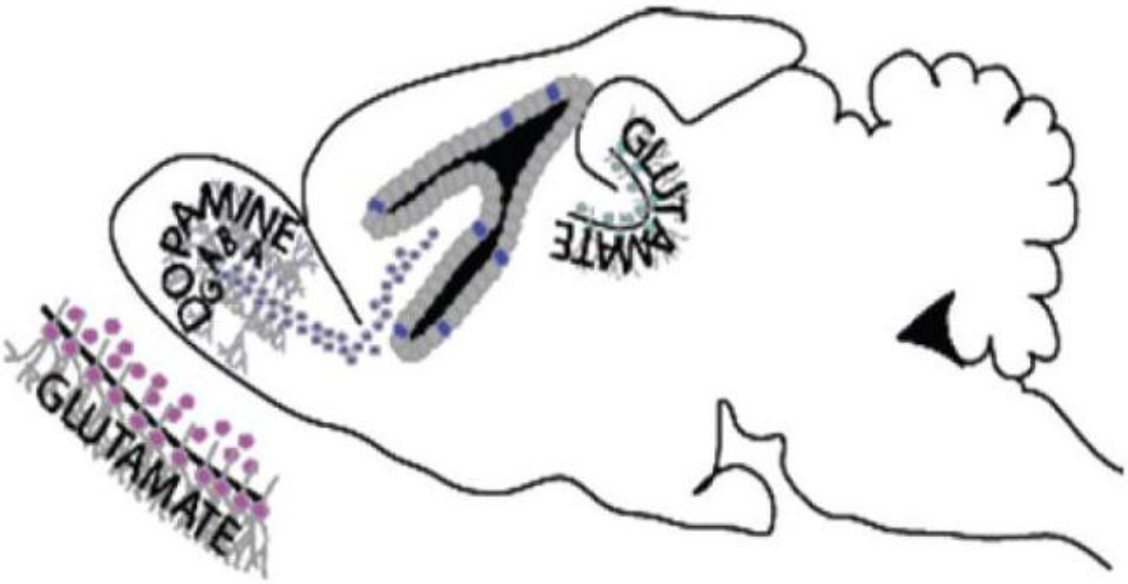

Figure 1-1. Cartoon of a rat brain showing hippocampal and olfactory bulb neurogenesis. NPCs that reside in the subgranular zone between the hilus and granule cell layer divide producing daughter cells. Many of these daughter cells migrate deeper into the granule cell layer and differentiate into granule neurons. NPCs that divide in the subventricular zone produce daughter cells that chain migrate through the rostral migratory stream into to the olfactory bulbs where they fully differentiate into dopaminergic periglomerular neurons and GABAergic granule neurons. Significant numbers of glutamatergic neurons are produced in the olfactory epithelium throughout life.

The regenerative capability of the brain has only been acknowledged and vigorously explored in recent decades. The goal of current stem cell research is to understand how to harness this regenerative capacity to restore function lost in disease or injury by replacing or rejuvenating dead or dying neural circuitry (Ormerod *et al.* 2008).

Adult Neurogenesis in the Hippocampus: identifying the mechanisms that control neurogenesis in the adult hippocampus or olfactory bulbs under normal

conditions can provide important insight about how to stimulate and control neurogenesis in extra-neurogenic regions. The hippocampus is an excellent natural model for studying the mechanisms that regulate adult neurogenesis for several reasons. First, thousands of new granule neurons are spontaneously added to the granule cell layer each day through life (Cameron & McKay 2001). Second, neurogenesis (the production, fate choice and survival of new neurons) can be modulated by a number of intrinsic and external factors like hormones (Ormerod *et al.* 2004), stress (Tanapat *et al.* 1998), inflammation (Monje *et al.* 2003), tobacco smoke (Bruijnzeel *et al.* 2011), and even hippocampus-dependent learning (Gould et al., 1999). Third, many behavioral tasks such as the water maze (Kempermann & Gage 2002, Ormerod *et al.* under review, Ormerod et al. 2004), radial arm maze (Clelland *et al.* 2009), Barnes maze (Raber *et al.* 2004), object recognition task (Bruel-Jungerman *et al.* 2005), eye-blink conditioning task (Wang *et al.* 2010), contextual conditioning task (Winocur *et al.* 2006), and inhibitory avoidance task (Jahanshahi *et al.* 2011) are sensitive to hippocampal integrity and can be used to assess the effects of modifying neurogenesis. For example, we and others have shown that rodents with rates of neurogenesis that are increased by daily exposure to an enriched environment or exercise outperform their controls in the Morris water maze (Wu *et al.* 2007, Speisman *et al.* in press, Jurgens & Johnson 2012). Importantly, experimental manipulations that influence distinct components of neurogenesis (NPC proliferation and the differentiation and survival of their progeny) may differentially influence hippocampus-dependent behavior. For example, physical activity increases the production of new cells (van Praag *et al.* 1999) while enrichment promotes their integration(Kempermann *et al.* 1997,

Speisman et al. in press). Stronger correlations between new neuron number and measures of learning and memory can be detected following enrichment versus physical exercise.

Investigators typically employ DNA labeling strategies to study the effects of an experimental manipulation on NPC proliferation or the differentiation and survival of their progeny. Dividing cells incorporate bromodeoxyuridine (BrdU) and other thymidine analogues into their DNA during the synthesis phase of mitosis instead of thymine (Miller & Nowakowski 1988). If an animal is euthanized within hours of BrdU injection, the experimenter can evaluate the number of dividing NPCs. Using this approach, investigators have revealed that NPC division in the adult hippocampus is complete within ~18h and that ~10,000 new cells are added to the adult rat hippocampus each day (Cameron & McKay 2001). If an animal is euthanized days or weeks after BrdU injection, the experimenter can examine whether the new BrdU+ cells express cell type specific proteins immunohistochemically (i.e. neuronal or glial in the brain; see Figure 1-2). Using this approach, investigators have shown that ~50% of the new cells produced survive 4 weeks and of the surviving cells ~ 80% are neurons expressing mature neuronal markers such as neuronal nuclei (NeuN; for example Cameron & McKay 2001).

The recent development of antibodies against often transiently-expressed early immature neuronal proteins, such as prospero homoebox protein-1 (Prox-1; Misra *et al.* 2008) and doublecortin (DCX; Brown *et al.* 2003) has permitted investigators to better understand the stages of neuronal maturation undertaken by many new hippocampal cells and investigate the effects of experimental variables on those stages. NPCs are

referred to as Type 1, Type 2a, Type 2b and Type 3 as they transition through these stages of neuronal maturation.

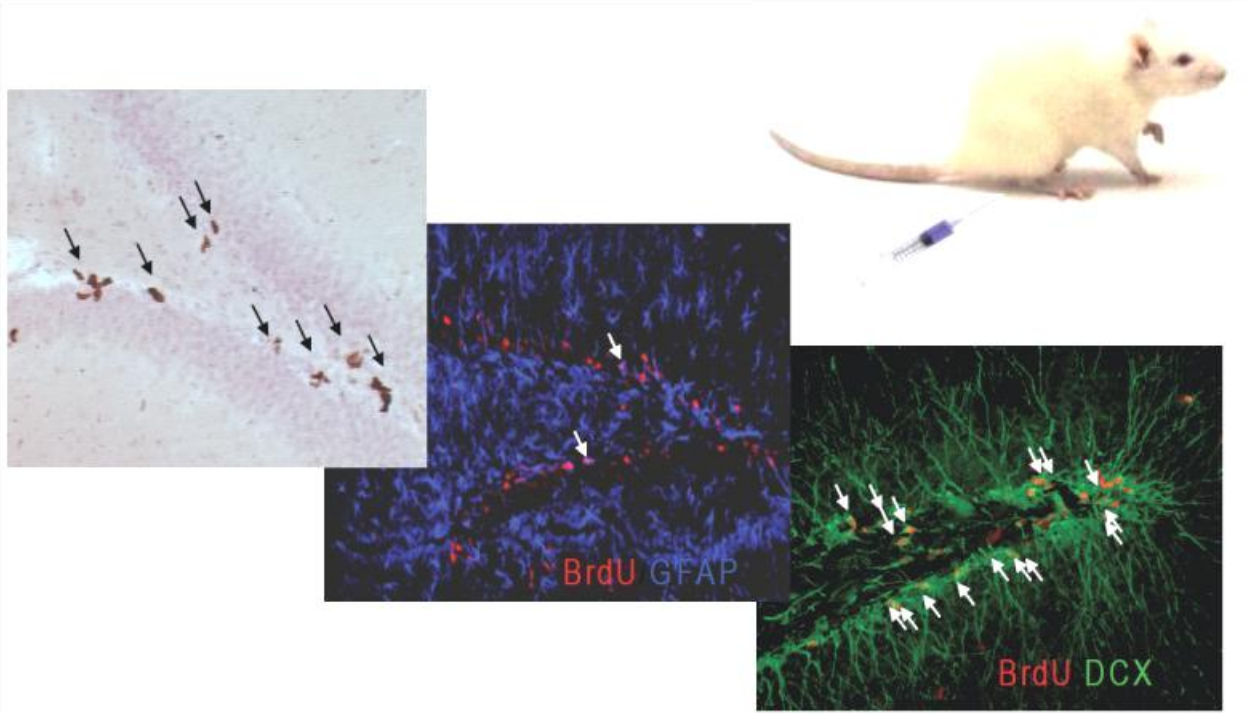

Figure 1-2. Neurogenesis in the dentate gyrus and subventricular zone. Use of Brdu marker injected in mice in order to label dividing cells and examples of himmunohistochemical staining of progenitors expressing the glial marker GFAP or the immature neuronal marker DCX. [Figure courtesy of Dr. Ormerod B.K.].

The most naïve 'Type 1' proliferating NPCs in the hippocampal SGZ are most abundantly observed within a few hours of BrdU injection. They morphologically resemble radial glia with triangular shaped soma. These cells express glial fibrillary acidic protein (GFAP; Debus *et al.* 1983, Gould *et al.* 1999), the neural progenitor marker nestin (Fukuda *et al.* 2003, Lendahl *et al.* 1990) and the transcription factor sex determining region-Y box 2 (Sox2; Abrous *et al.* 2005, Uwanogho *et al.* 1995). Type 1 cells are thought to produce intermediate Type 2a cells that retain expression of transcription factors, such as Sox2 and nestin but also express the proneural transcription factor Mash 1 (within hrs of BrdU incorporation; Itoh *et al.* 1997). Within

~24h of BrdU incorporation, neuronally committed Type-2b intermediate progenitor cells phenotypes emerge that retain low-level NPC marker expression and lose Mash-1 but gain Prox-1 expression. Type-3 neuroblast phenotypes then emerge that lose NPC marker expression, retain Prox-1 expression, gain transient DCX expression, and then permanent NeuN expression while maturing into functional granule neurons (von Bohlen Und Halbach 2007; see Figure 1-3). Note that the characteristics of these cells vary slightly depending upon the markers combinations employed and the durations after BrdU injection that the cells are phenotyped after.

New neurons extend an axon to synapse with CA3 region pyramidal neurons between 4 and 10 days after birth (Hastings and Gould, 1999), and those new neurons surviving 4 weeks (~50% of all new cells produced) are thought to be relatively permanent (Cameron & McKay 2001). New cells that do not survive appear to undergo apoptosis and are phagocytosed by resting microglia and potentially migrating neuroblasts (Sierra *et al.* 2010, Lu *et al.* 2011). New neurons exhibit mature morphologies after ~ 1month and mature electrophysiological profiles after ~ 2 months (van Praag *et al.* 2002). Importantly, these stages of neuronal maturation are refined with the development of new markers and we dispose of less information about the maturation of new hippocampal oligodendrocytes and astrocytes. This is likely because far fewer astrocytes and oligodendrocytes are produced from neural progenitors in the adult hippocampus and our interest is focused principally on the treatment of diseases that seek to replace neurons.

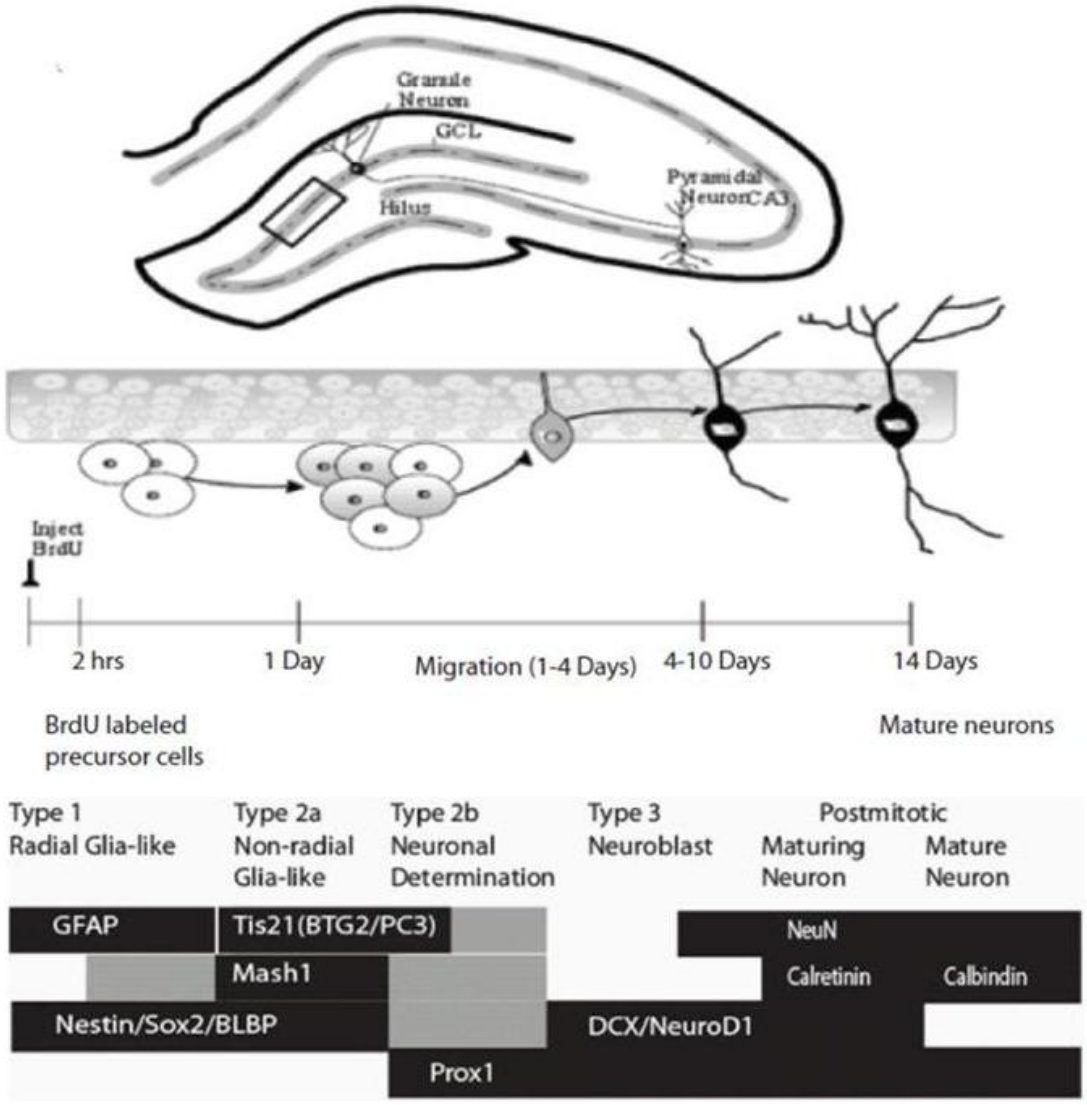

Figure 1-3. Dentate gyrus and different development stages of neural progenitors showing different marker expression associated with the evolutionary stage of neural progenitors. [Figure courtesy of Dr. Ormerod B.K.]

Importantly, some experiments employing single versus multiple BrdU injections revealed an effect of neural inflammation on neurogenesis. In particular, the use of one injection allowed them to label mitotic cells during a shorter period of time while multiple injections gave data on the overall production of new cells during a bigger time window.

These experiments revealed an effect on neural differentiation but not in new cell proliferation due to induced inflammation (Asokan & Ormerod 2009).

Microglia

Neuroinflammation is thought to be elaborated primarily through the activity of the central nervous system's (CNS) resident macrophages, microglia. Microglia were first described by Pio del Rio Ortega in the early 1930s (del Rio Hortega 1930) as the brain's immune cells. Recently, the scientific community has become intensely interested in the role of microglia in brain repair and cognition. This interest has been stimulated by the findings that microglia are activated by brain injury, aging (Sheffield & Berman 1998), and neurodegenerative diseases, which are all associated with cognitive impairments (Itagaki *et al.* 1989, Oyebode *et al.* 1986). Importantly, microglia activation accompanies the syndromes associated with cognitive impairment and may or may not contribute to the cognitive impairment per se. In fact, a PubMed search on "microglia" produces 9,500 articles published just in the last 10 years.

Microglia are of myeloid origin and constitute between 5 and 10 % of cells in the CNS (Polazzi & Monti 2010). Microglia are thought to become incorporated into the CNS during development and then around birth, when vascularization of the CNS is already complete there is a massive increase in their number (Richardson *et al.* 1994). Like other adaptive immune cells, microglia react to stimuli by becoming "activated" to produce and secrete pro-and anti-inflammatory molecules and can even phagocyte dead cells and debris. We are just beginning to understand the unique and numerous roles that brain microglia play. An important role might be in particular played by microglia in the regulation of neurogenesis, and healthy microglia are thought to be critical for maintaining plasticity in the healthy brain (Cullheim & Thams 2007).

Function of Microglia in the Healthy Brain and Pathological Conditions

An emerging picture suggests that resting microglial cells maintain homeostasis in the healthy brain. Microglial housekeepers continually clean their parenchymal environment of metabolic byproducts and dead and dying cell debris (Wake *et al.* 2009, Nimmerjahn *et al.* 2005). Recent work *in vivo* live cell imaging work has shown that resting microglia may also monitor and modulate neuronal communication; they appear to prune some neuronal synapses after contacting them with their processes (Nimmerjahn et al. 2005, Wake et al. 2009). While synaptic pruning has been a long-studied form of plasticity in the neurosciences, this is a novel and interesting mechanism by which microglia may control the process. In early development, microglia also stimulate angiogenesis in the retina and postnatally resume this role in ischemic retinopathy (Checchin *et al.* 2006). With prolonged hypoxia, there is typically a reduction in microglia and abnormal neovascularization, and if microglia are excluded from the ischemic retinal environment, there is reduced angiogenesis, which can be restored by replacing microglia. These data suggest that resting microglia may play a greater role in normal CNS function than previously thought.

The role that microglia play at any one point in time appears dependent upon the status of cells within their region of the network. Currently, a large amount of research effort is being targeted at revealing this network and the signaling pathways involved. Microglia express cytokines that act on receptor proteins expressed by astrocytes, neurons and vascular cells. For example, chemokines produced by astrocytes during multiple sclerosis have shown to engender microglia activation supporting the inflammatory response (Tanuma *et al.* 2006). In addition, they express receptor proteins for factors secreted by cells in their environment. For example, microglia react to IL-4

released by T-helper and exert a neuroprotective role in the controlled immune reaction (Butovsky *et al.* 2006). Not only do microglia affect neurons, they can influence astrocytic production of neurotransmitters, such as glutamate and they can respond to factors that astrocytes and neurons secrete in response to stimuli, such as viruses (Ovanesov *et al.* 2008). Therefore microglia are activated though communication within their network in response to different stimuli indicating alteration of the homeostasis in the parenchyma. For example, microglial activation accompanies pathogen invasion, injury and disease, but whether this response is beneficial or detrimental is under intense debate (Streit & Xue 2009). Neuroinflammation is a complex process that involves CNS parenchyma cells perivascular macrophages and peripherally released factors. Although their precise role in battling injury and disease is not completely clear, we are beginning to understand roles that microglia play while in different stages of activation.

Microglia are typically described as being in resting or activated states. These states have been described in detail in an excellent review published by Graeber and Streit (Graeber & Streit 2010) Among microglia, 4 activation states or phenotypes can be readily distinguished using modern imaging and phenotyping techniques(see Figure 1-4). Microglia can be resting, activated non-phagocytic, activated phagocytic and dystrophic. Microglia are typically observed in the healthy CNS. The term resting may misrepresent the housekeeping functions that microglia undertake in the healthy CNS (see Wake et al. 2009). In addition to keeping their environment clean and perhaps monitoring synaptic communication, they are attuned to detect possible homeostatic alterations through signaling in their networks. Resting microglia are moderately

ramified. In the face of neuroinflammation not associated with robust cell death, microglia become activated but non-phagocytic microglia. Activated non-phagocytic microglia become more highly branched and hypertrophic while producing the pro- and anti-inflammatory cytokines thought to be necessary for re-establishing homeostasis in the CNS. Phagocytic microglia thought to be activated by significant cell death and blood brain barrier (BBB) damage exhibit reduced branching and hypertrophy. Phagocytic and non-phagocytic activated microglia are thought to proliferate in order to increase their numbers for a more effective response and then die apoptotically. Finally, a dystrophic phenotype is exhibited by microglia in the aging CNS or after chronic over-activation, which can occur with neurodegenerative pathologies such as Alzheimer's disease. Irregularly shaped dystrophic microglia are highly branched and often fragmented as they undergo 'accidental' (rather than apoptotic) cell death (Streit & Xue 2009). These 4 phenotypes are currently considered the standard for classifying microglia phenotypically. However, microglia may profoundly alter their cytokine profiles while in a particular morphological state.

In addition to examining the activation state of microglia in different experimental contexts morphologically, their activation state can be detected using protein biomarkers. Ionized calcium binding adaptor molecule- 1 (IBA-1) is a protein expressed throughout the cell body and processes of circulating monocytes/macrophages and the CNS-resident counterpart microglia (Xue *et al.* 2010, Imai *et al.* 1996). If an experiment is associated with BBB damage, additional markers are required to differentiate between infiltrating monocyte/macrophages and resident microglial cells. Cluster of Differentiation 68 (CD68 or macrosialin) is expressed at low-levels on all

monocytes/macrophages and microglia, as well as other cell types but its expression increases with phagocytosis in monocytes/macrophages and microglia (Xue et al. 2010, Micklem *et al.* 1989).

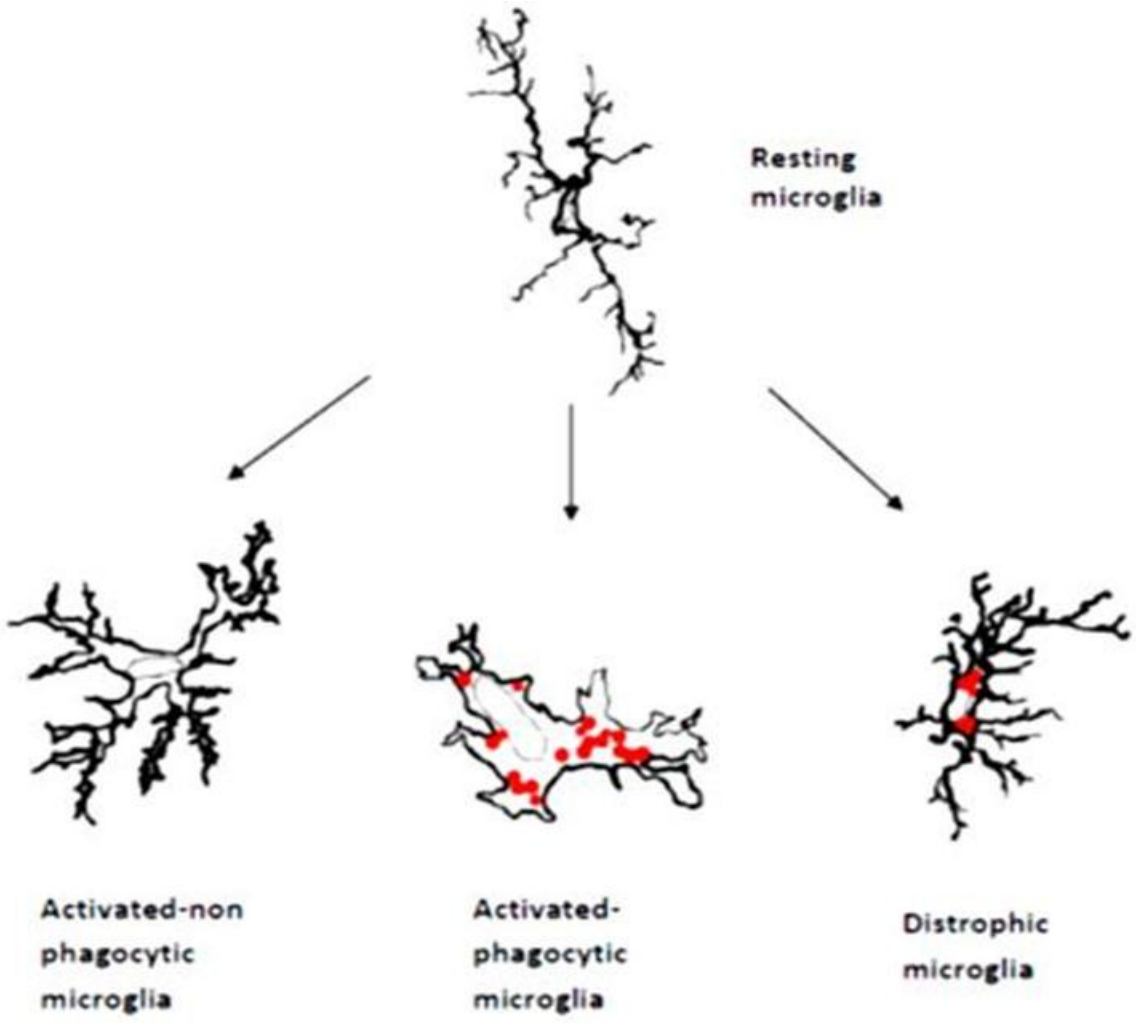

Figure 1-4. Microglia phenotypes. In normal conditions, microglia assume a resting phenotype, when stimulated activated-non phagocytic or activated-phagocytic phenotype can be expressed, both these phenotypes present hypertrophic cell body and in phagocytic phenotype a reduction of branching is present. Dystrophic microglia present an irregular shape that might be related to aging of microglia and partial loss of functionality. CD68 marker represented in red is present in activated phagocytic and dystrophic phenotypes. [Adapted from Streit and Xue 2009.Life and death of microglia.(pag. 373 Fig.1)]

Cluster of differentiation molecule 11β (CD11β) is expressed in activated phagocytic or non-phagocytic monocytes/macrophages and microglia (Zhang & Plow

1999, Smith *et al.* 1989). We employed both markers to phenotype microglia in the experiments conducted for this thesis.

Microglia as Central Nervous System Immune Cells

Streit (2002) elegantly summarizes how external stimuli could activate microglia resident to the brain. Because the skull, meninges and BBB protect the CNS from injury and invading pathogens dogma has been that the CNS is immunologically privileged, or protected, from communication with the peripheral immune system. Research in recent years has shown that the BBB separates the circulating immune system from the recently recognized neuroimmune system. In fact, until recent years, the CNS was through to be privileged from the effects of the peripheral immune system. The prevalent rationale was that aggressive peripheral immune cells, such as activated leukocytes, may damage fragile neuronal cells.

We now know that the CNS contains a robust neuroimmune system that communicates through several pathways with the immune system. For example, the vagus nerve can sense peripheral immune activation to brain regions that include the hypothalamus and the brainstem (Fleshner *et al.* 1995, Mravec 2011). Further evidence that there is dialogue between the central and peripheral immune systems comes from facial nerve axotomy, which can activate microglia (Nakajima *et al.* 2005). This is an evidence of a dialogue between neurons and microglia and this communication can trigger microglia activation and at the same time microglial cells could have a role in the regeneration of the nerve. Some peripheral cytokines, such as IL1-β and TNF-α can diffuse through the BBB (Banks *et al.* 1991, Banks *et al.* 2001) while others are actively transported by proteins through circumventricular organs. Cells of the BBB that include endothelial cells, perivascular smooth muscle cells and ependymal cells express

receptor proteins for circulating cytokines that induce the production of cytokines when they are activated(Liu *et al.* 2012). Microglia recognize and engulf immunoglobulin and thrombin produced by BBB damage and extravasating peripheral immune cells to reduce their concentrations in the brain (Möller *et al.* 2000). These findings suggest that microglia actively surveil not only CNS status, but the status of the peripheral immune system.

In addition to their well-known phagocytic roles, microglia can also behave as antigen-presenting cells (APCs; Streit 2002). However, leukocytes/lymphocyte infiltration into the CNS only occurs in some cases (with multiple sclerosis or HIV infection), so the functional outcome of their APC role is unclear (Streit 2002). They could be presenting antigen to T-lymphocytes located in the perivascular space, although a role for responsive T-lymphocyte secretory factors in the injured or diseased brain is also unclear (Graeber & Streit 1990). This hypothesis is supported from the observation that microglial cells are usually located in the parenchyma and not always close to vessels making it harder for them to present antigens to circulating cells. In the described experiments, we examine the negative effect of microglia on neurogenesis during a neuroinflammatory assault, but microglia likely exert positive effects on neurons during the assault and are likely beneficial to young neurons while resting.

Microglia and Neuroprotective / Neurotoxic Role during Neuroinflammation

The neuroprotective or neurotoxic role of microglial cells has recently been at the center of debate. Activated microglial cells appear detrimental to the production of new neurons (Monje et al. 2003). In the adult rodent hippocampus, the number of new neurons produced in the hippocampus correlates negatively with the number of activated microglia following experimental manipulations that induce neuroinflammation,

such as LPS administration or targeted γ-irradiation. Cultured NPCs exposed to LPS-stimulated microglia produce new cells that are significantly less likely to acquire a neuronal phenotype, potentially through IL-6 activity. Of course, peripheral LPS-induced neuroinflammation can impact many cell subtypes that could produce factors that ablate neurogenesis. However, activated microglia remain key suspects based upon published reports.

On the other hand, activated microglia appear to protect mature neurons. For example, ischemia-induced cell death in the CA1 region of the hippocampus does not spread to the CA2 region of the hippocampus, which would be expected if activated microglia produced diffusible molecules that were detrimental to neuronal health (Streit *et al.* 1992). In the case of facial nerve axotomy, microglia ensheath the region of the axon around the lesion, remove potentially excitotoxic molecules that are secreted following injury and deliver growth factors associated with repair (Streit 2002). After an acute neuroinflammatory challenge, microglia phagocytose debris and dying neurons in a process referred to as “facilitative neurotoxicity” by Streit, which resembles the cellular euthanasia of neurons that would have otherwise undergone cellular death by microglia. This evidence supports a protective role of microglia on neurons after acute inflammation. During chronic or pathological inflammatory conditions, microglial cells appear pseudo-activated or senescent/dystrophic. Here they appear to lose their ability to maintain homeostasis by performing their normal functions and there is a dangerous accumulation of neurotoxic molecules that may contribute to cognitive decline (Streit & Xue 2009). Therefore, completely suppressing microglial activities may compromise the health of neurons and neuronal networks that survive disease or injury.

In fact, resting and even activated microglial could facilitate adult hippocampal neurogenesis in a number of ways. They could phagocytose the 50% of new cells that fail to thrive (Oppenheim 1991). Microglia could also directly regulate new cell fates. For example, neurogenesis is decreased in immune-deficient mice that lack microglia and that exhibit impaired spatial learning and memory abilities (Ziv *et al.* 2006). Microglia may eliminate potentially toxic debris that may compromise the health of young neurons, such as excess glutamate (Streit 2002). They may also control the synaptogenesis that occurs in order to form correct neural connections with their target CA3 pyramidal neurons (Wake et al. 2009, Wake *et al.* 2012). However, understanding how to control microglial in a neuroinflamatory environment appears critical if neurogenesis is required. Given that the goal of many neural engineering strategies is to replace dead or dying circuits in the diseased or injured brain discovering the neuroinflammatory molecules that ablate neurogenesis is critical.

Neuroinflammation and Neurogenesis

Many intrinsic, extrinsic and epigenetic factors have been identified that influence hippocampal neurogenesis in adult mammals (Ge *et al.* 2007). Some factors, such as hormones and cytokines are thought to pass through the blood brain barrier from circulation to either promote or inhibit neurogenesis (Ormerod et al. 2004, Czirr & Wyss-Coray 2012). Furthermore, signals generated by cells in the neurogenic niche that include astrocytes, microglia, endothelial cells and other neurons may influence the division of NPCs and the differentiation and survival of their progeny (Suzumura *et al.* 2006). Astrocytes, which are in contact with all cells in the neurogenic niche, secrete molecules and form gap-junctions trough whom they could regulate the differentiation of precursors in normal conditions and specifically during the inflammatory process

(Ekdahl 2012). Mature neurons as well, may use signals to modulate neurogenesis by releasing neurotransmitters and a modification in this effect might be seen in case of an inflammatory stimulus that could alter neurotransmitter release. Microglia cells are located in close proximity with progenitors and are thought to be the primary mediators of the neuroinflammatory response that ablates neurogenesis.

Lipopolysaccharide is an endotoxin that is a part of the outer membrane of gram-negative bacteria (Warner *et al.* 1975). LPS is involved in normal bacterial inflammation and can be found in the blood of patients infected with gram-negative bacteria (Levin *et al.* 1970). The pro-inflammatory role of LPS was confirmed in studies where anti-endotoxin was used to reduce the concentration of LPS in patients during septic shock (Gaffin & Lachman 1984). LPS, when injected peripherally causes an increase in the production of inflammatory cytokines and consequently induces an inflammatory condition in the brain that is comparable to many types of infections in the human body (Turrin *et al.* 2001). We stimulated an inflammatory and then neuroinflammatory response in our experiments with intraperitoneally injected LPS. We used LPS because it is well-tolerated by rodents and its effects on neurogenesis at the dose employed well-established. Doses of 1mg/kg in rats and 5mg/kg in mice activate microglia and ablate neuronal differentiation among the NPC progeny without affecting NPC proliferation (Asokan & Ormerod 2009, Monje et al. 2003).

In rats, a single peripheral 1mg/kg injection of LPS ablated hippocampal neurogenesis (Monje et al. 2003). Peripheral LPS stimulated a neuroinflammatory response because the density of ED1 (CD68) positive microglia was elevated at the end of one week, and in fact, the number of activated microglia correlated negatively with

the number of new neurons. In addition, the non-steroidal anti-inflammatory drug (NSAID) indomenthacin prevented peripheral LPS from activating microglia and from ablating hippocampal neurogenesis. Even when injected directly into the hippocampus, LPS appears to ablate neurogenesis by stimulating microglia (Ekdahl *et al.* 2003, Monje et al. 2003). After a 4 week-long intracortical delivery of LPS hippocampal neurogenesis was reduced in adult mice unless they were treated with the tetracycline minocycline, which protected neurogenesis and microglial activation from the effects of centrally administered LPS. These data suggest that spontaneous neuron production, and likely neuron production using transplantable cell strategies, is compromised by a microglial-mediated neuroinflammatory response.

A recent study in our laboratory revealed that LPS stimulates the production at least 32 cytokines in the hippocampus of adult mice, that return to baseline within ~96h of LPS injection (Asokan & Ormerod 2009). Therefore we examined microglial activation, based upon morphology and the expression of activation markers over the first 96h after LPS injection. Also, Monje and colleagues (Monje et al. 2003) showed that microglia were activated for at least a week after LPS injection and preliminary data in our laboratory suggests that they may, in fact, remain activated for several weeks after LPS injection. If microglia remain activated for several weeks after a peripheral immune challenge, then the hippocampal neurogenesis may be ablated for several weeks, which would presumably be associated with serious cognitive decline. Here we also examined the longevity of the effects of a single peripheral LPS injection on neurogenesis and microglial activation in the hippocampus of adult mice.

CHAPTER 2
MATERIALS AND METHODS

Animals

All mice used in this study were treated in accordance with relevant National Institutes of Health and University of Florida guidelines regarding the use of animals for research. Animal facilities and experimental protocols complied with the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) policies and were approved by the University of Florida Institutional Animal Care and Use Committee. Every effort to limit the number of animals and to reduce their discomfort was made. CB57BL/6 female mice 8 weeks old (n=76;Taconic, United States of America) were housed in groups of 5 in cedar bedding-lined shoebox cages and had free access to autoclaved water and food (Prolab Mouse Chow, PMI Nutrition International,St. Louis,MO). The colony room that they were housed in was set on a 12:12h light:dark cycle (lights on at 6am) and was maintained at 23°C.

Brdu Preparation

The thymidine analogue bromodeurixidine (BrdU; Sigma Aldrich, St. Louis, MO) is incorporated into the DNA of dividing cells up to 4 hours after intraperitoneal administration. BrdU was dissolved in a concentration of 10mg/ml and injected intraperitoneally at a dose of 50mg/kg to label dividing cells, considering the average weight of treated mice was 20g mice were correspondingly treated with a volume of 100µl/mouse. This dose effectively labels cells and appears safe in adult rodents (Ormerod & Galea 2003, Kolb *et al.* 1998).

LPS (Lipopolysaccharide) Preparation

Bacterial lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO) a known immunogenic molecule present in the outer membrane of gram-negative bacteria (Warner et al. 1975) was dissolved in sterile 0.9% saline solution at the concentration of 0.5 mg/ml. A dose of 5mg/kg (or 2.5ml/kg) of LPS was injected intraperitoneally on Day 0. All mice were injected subcutaneously at 24h after LPS to prevent dehydration.

Anesthetic Preparation

A ketamine/xylazine injection was used in order to deeply anesthetize mice. Doses of 90 mg/kg ketamine and 10mg/kg of xylazine were diluted in 0.9% saline solution. Effectiveness of anesthesia was confirmed by lack of response to a foot pinch.

Paraformaldehyde Preparation

Granular paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pennsylvania) was dissolved to 8% in distilled water heated to 60°C. The pH of the solution was adjusted to 7.4 with sodium hydroxide (NaOH) and diluted with 0.2M phosphate buffered saline. The solution was chilled to 4°C and then used to both perfuse the mice transcardially and to postfix the brains overnight.

Experiment 1

Experiment 1 evaluated the short-term effects of LPS injection on microglial morphology and activation state. One week after arrival half of the animals were treated with lipopolysaccharide to induce transient illness (Day 0) and the other half with freshly prepared sterile saline solution to control for injection stress (n=18 per group). Equal numbers of LPS- and saline-treated mice were anaesthetized with ketamine/xylazine either 5h (n=5 per group), 24h (n=4 per group), 48h (n=4 per group) or 96h (n= 5 per group) after treatment by transcardial perfusion. To test the short-term effect of LPS

administration on microglial activation we analyzed co-expression of IBA-1 with CD11β and CD68 and measured microglia soma areas.

Experiment 2

Experiment 2 measured the effects of LPS administration on neurogenesis and on microglial activation weeks after infection. Mice were injected intraperitoneally with LPS (5mg/kg; n=20) or an equivalent volume of 0.9% saline (n=20). LPS- and saline-treated mice were injected once with BrdU 5h after treatment and perfused one week later (n=5 per group). Separate groups of LPS- and saline-treated mice were injected 3 times with BrdU beginning either 1 week, 2 weeks or 3 weeks after treatment and perfused one week later (n=5 per group). At the end of the one-week survival period after BrdU, the mice were anaesthetized deeply using the ketamine/xylazine mixture. Once anesthesia was confirmed by lack of response to a foot pinch, the mice were killed by transcardial perfusion New cell phenotypes were evaluated by examining the proportion of $BrdU^+$ cells that expressed the neuronal proteins DCX and/or NeuN expression and the activation state of microglia was evaluated by examining the proportion of IBA-1^+ cells that co-expressed CD11β and/or CD68 and by examining their cell body areas.

Histology

In Experiments 1 and 2, brains were extracted rapidly after perfusion, post-fixed in paraformaldehyde for 24h then transferred to 30% sucrose solution until they were equilibrated (sank, ~4 days) The brains were sectioned at 40 μm intervals through the rostral-caudal extent of the hippocampus on a freezing stage microtome (American Optical Corporation, Buffalo, NY) starting about 1mm caudal to the bregma till about 3.5mm caudal to the bregma (Paxinos *et al.* 1980). These coordinates ensured that the entire portion of the hippocampus containing the neurogenic dentate gyrus was

collected. Series of every 12th section were stored in a cryopreservative solution (30% ethylene glycol, 30% glycerol and 30% phosphate buffer) at -20 C until stained immunohistochemically.

Immunohistochemistry

Section were prepared as described previously (Ormerod et al. 2004) and stained using 1 of 3 antibody combinations. To detect phagocytic microglia, sections were incubated overnight at 4C°in rabbit anti-IBA-1 (Imai et al. 1996; 1:1000;Wako, Richmond, VA), and rat anti-CD68 to detect phagocytic cells (Xue et al. 2010, Micklem et al. 1989; 1:500; ABD Serotec, Raleigh, NC). To detect activated microglia, sectioned were incubated overnight at 4C°in rabbit anti-IBA-1 (1:1000;Wako, Richmond, VA) and rat anti-CD11β to detect activated cells (Smith et al. 1989; 1:100; BD Bioscience, San Jose, California). To confirm the neuronal phenotype of new cells, sections were incubated over night at 4C° in mouse anti-Neuronal Nuclei (NeuN; Millipore, Billerica, MA; 1:500) to detect mature neuronal protein (Cameron et al. 1993, Ormerod *et al.* 2003) and goat anti-doublecortin (DCX; Santa Cruz Biotechnology, Accurate, NJ; 1:500). to detect an early stage of neural progenitor differentiation (Chelly 1998) . The next day, the sections were incubated for 4 hours at RT in the appropriate FITC- and Cy5-conjugated fluorescent IgG conjugated secondary antibodies (1:500; Jackson Immunoresearch, West Grove, PA), rinsed, denatured in 2 M HCl for 30 min at 37C° and then incubated overnight in anti-mouse BrdU (concentration 1:500; AbD Serotec, Raleigh, NC) at 4C° and then Cy3-conjugated anti-rat IGg secondary antibody to reveal cells that had incorporated BrdU. All sections were incubated in DAPI to reveal cellular nucleus for 10 min (1:3000; Calbiochem, EDM Millipore, Billerica, Massachusetts) before being cover-slipped under PVA-DABCO.

Data Analysis

Evaluation of Microglia Phenotype

To evaluate microglia phenotype confocal images were taken using Zeiss LSM 710 fully spectral Laser Scanning Confocal Microscope (with 405, 440, 488, 532, 635 laser lines) with a 20x (5x and 1x digital zoom) and 40x (1x digital zoom) oil immersion objective through the z-plane. Laser intensities were kept below 11% and gain/offset values were set on control sections. IBA-1 positive cells, identified as microglia, were counted if the nucleus was unambiguously labeled with DAPI and if it was localized in the granular layer and the SGZ, that consists in a 50µm layer between the granular layer and hilus (Palmer *et al.* 2000).

Microglia were considered activated if co-labeled with CD11β or CD68. The presence of CD11β or CD68 marker was evaluated on a least 5 z-plane sections through each IBA-1^+ cell and then the proportion of activated microglia (IBA-1^+ versus IBA-1/CD11β^+ cells) or phagocytic (IBA-1 versus IBA-1/CD68$^+$ cells) was assessed. Because all IBA-1^+ cells expressed low-level CD68, we confirmed that CD68 pixel intensities co-localized with IBA-1 in the hippocampi of saline and LPS-treated mice were similar using the Zen Zeiss Confocal Microscope Software threshold tool (see Figure 2-1).Microglial activation was also evaluated by quantifying cell body areas (i.e. hypertrophy). The largest cell body diameter on each of the 5 z-plane sections taken through each of > 30 IBA-1^+ cells per animal was selected and the area of the cell determined using the Zen Zeiss Confocal Microscope processing software perimeter tool.

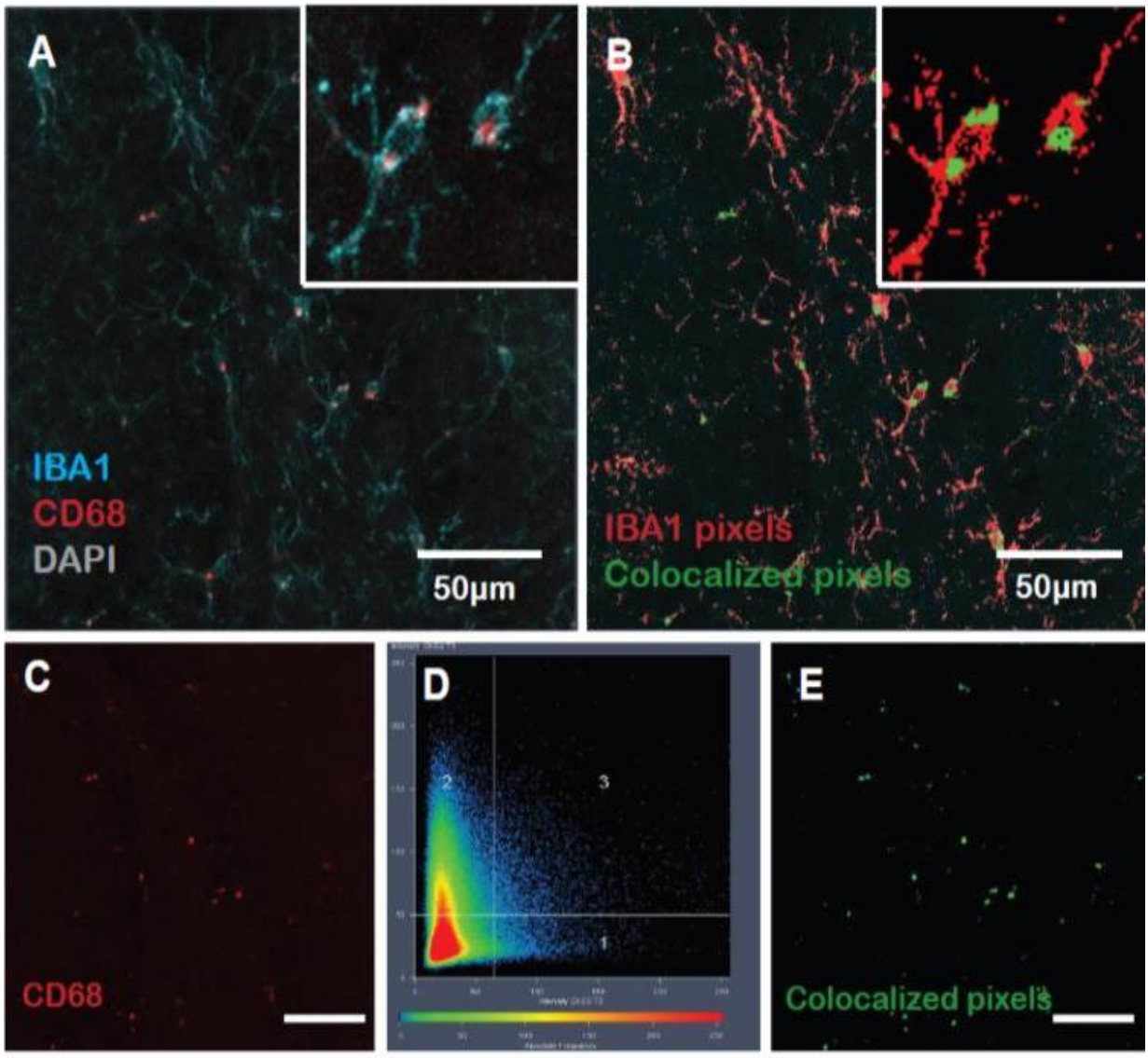

Figure 2-1. Co-localization parameters were chosen in order to minimize false co-localization of IBA-1/CD68 markers. A) Actual picture of microglia cells (light blue: IBA-1) co expressing CD68 (red: CD68). B) Identification of IBA-1 pixels over threshold intensity (red) and CD68 (green) co-localized pixels. D) Scatter plot of pixel intensity, on the axis are represented IBA-1 and CD68 channels; area 1 represents the area above threshold for CD68 but below for IBA-1 indicating the presence of CD68 staining not co-localized with IBA-1 and region 2 vice versa. Region 3 of the scatter plot represents the area of co-localized pixels. C) Actual picture of CD68 marker and E) representation of co-localized CD68/IBA-1 pixels (green) and CD68 pixels over threshold but not co-localized (blue).

Evaluation of New Cell Phenotypes

BrdU labeling was confirmed. A new cell was identified by the presence of BrdU marker evaluated going through the z-planes (at least 5 planes) with a 20x objective. A new cell was counted if the nucleus was unambiguously labeled with DAPI and if it was localized in the granular layer or in the SGZ. To identify phenotype of new cells, proportion of BrdU cells co-labeled with cell progenitor and neural specific markers (DCX, NeuN) was determined by scoring 100 positive cells, when possible. The proportion of $BrdU^+$, $BrdU^+/DCX^+$, $BrdU^+/DCX^+/NeuN^+$, $BrdU^+/NeuN^+$ was then calculated in order to have an indicator of the differentiation rate of progenitor cells.

Statistical Analysis

An analysis of variance (ANOVA) was used to verify the effect of the independent variables of treatment (Control and LPS) and time on the measures of microglia phenotype (proportion of CD11β, CD68 pixel intensity and cell body area) and neural differentiation (new cells phenotype). Newman Keul's post hoc tests was used to revealed group differences when appropriate. Differences were considered statistically significant for $p<0.05$.Statistical analyses were performed using STATISTICA software (StatSoft; Tulsa, OK). Values are expressed in graphs as mean ± SEM.

CHAPTER 3
RESULTS

Experiment 1: Peripheral LPS Does Not Stimulate Phagocytosis among Microglia

All IBA-1^{+} microglial cells expressed the phagocytic marker CD68 at low-level (see Figure 3-1). We therefore, confirmed that CD68 pixel intensities co-localized with IBA-1 were similar in the hippocampi of LPS-and saline-treated mice. An ANOVA confirmed that there was neither an effect of treatment ($F_{(1,16)}$=0.18; p=0.66) nor time after treatment ($F_{(3,16)}$=0.24; p=0.86) and these effects did not interact ($F_{(3,16)}$=0.24; p=0.86; see Figure 3-2). These data suggest that peripherally administered LPS did not stimulate phagocytosis in microglia within the first 4 days after LPS administration.

Peripheral LPS Stimulated an Activated Microglia Morphology

We quantified microglia soma areas to determine whether microglia exhibited activated but not phagocytic morphologies at various time points after peripheral LPS. An ANOVA exploring the effects of treatment (saline versus LPS) and time after treatment (5h, 24h 48h and 96h) found statistically significant effects of treatment ($F_{(1,16)}$=31.01*;* p<0.01) and time after treatment ($F_{(3,16)}$=8.57; p<0.01) and a statistically significant treatment x time interaction effect ($F_{(3,16)}$=3.99; p<0.05). Specifically, larger microglia were found in the dentate gyri of LPS-treated mice (49.64±1.93) versus saline-treated mice (42.41±0.82). Independent of group soma area increased with time after treatment such that they were larger at the 24 and 48h time points vs. the 5h time point (p<0.01). By 96h soma sizes were still larger than they were at the 5h time point (p<0.05) but not as large as they were at the 24h (p<0.05) and 48h (p<0.01) time points. As expected, this effect was mediated by LPS-induced changes in soma size. While the soma sizes of microglia in the control group were consistent across all time points

examined (p>0.49), they increased in the LPS-treated group by 24 and 48h relative to 5h (p<0.05) and tended to be higher at 96h, as shown in Figure 3-3.

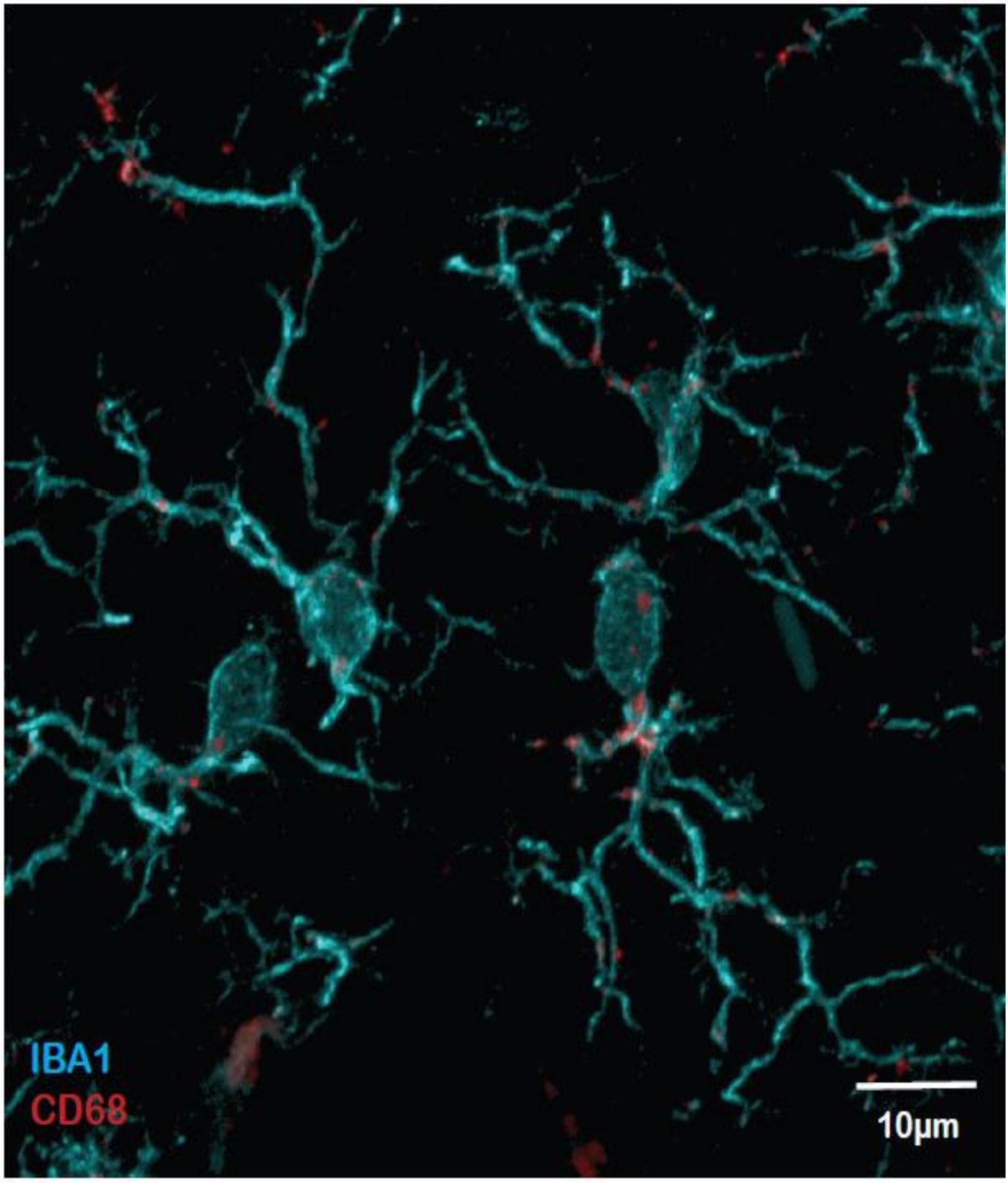

Figure 3-1.Low expression of CD68 is revealed in all microglia. Microglia identified with IBA-1(blue) and labeled with phagocytic marker CD68 (red).

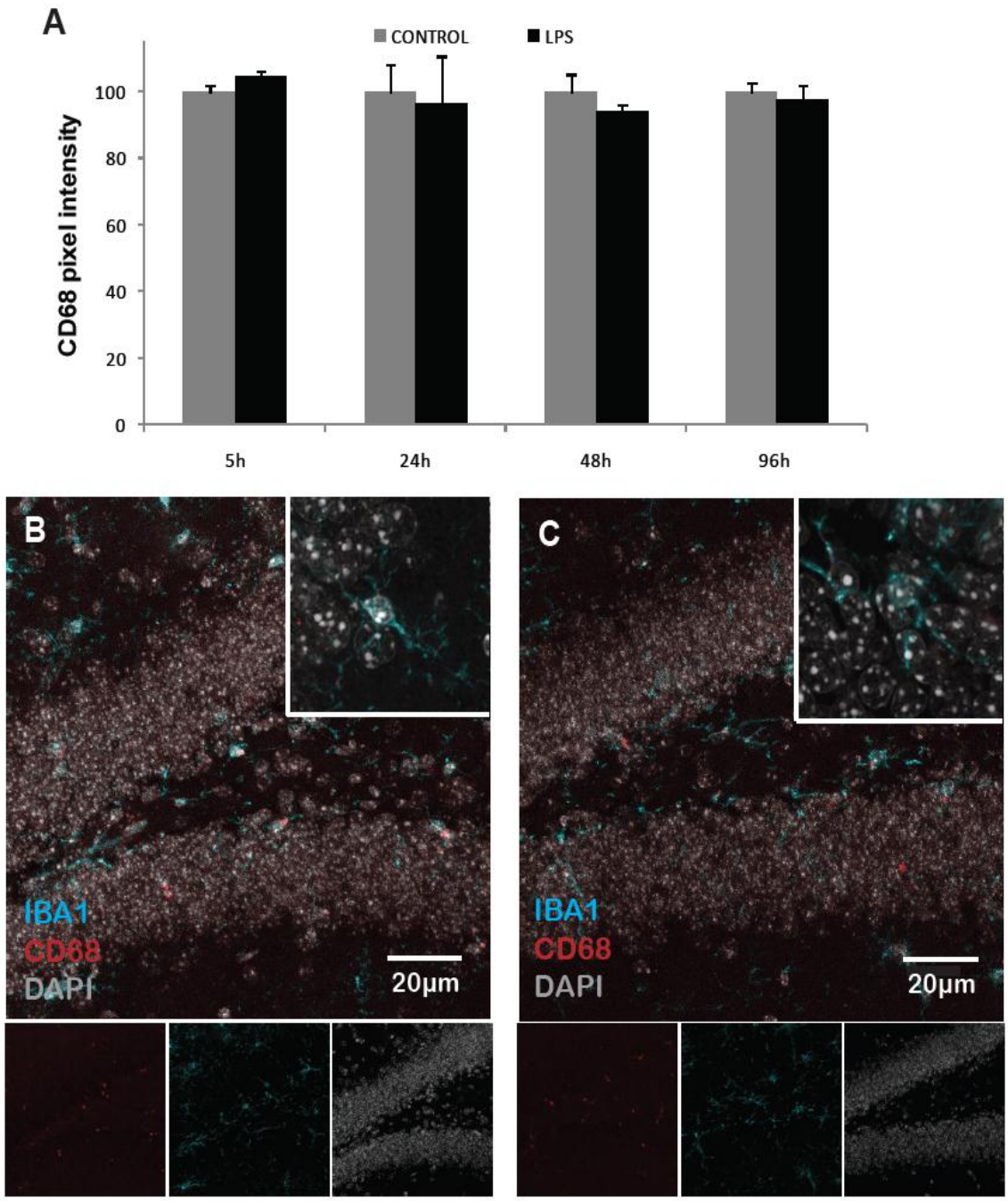

Figure 3-2. No microglial activation has been detected at 1 week and 4 weeks after LPS administration.A) Pixel intensity of co-localized CD68 reveals no phagocytic activity (horizontal axis: time; vertical axis; percentage of control pixel intensity). B and C) Area of cell body showing absence of hypertrophy of the cell body due to intraperitoneal LPS (horizontal axis: time; vertical axis: area of cell body expressed in μm^2).

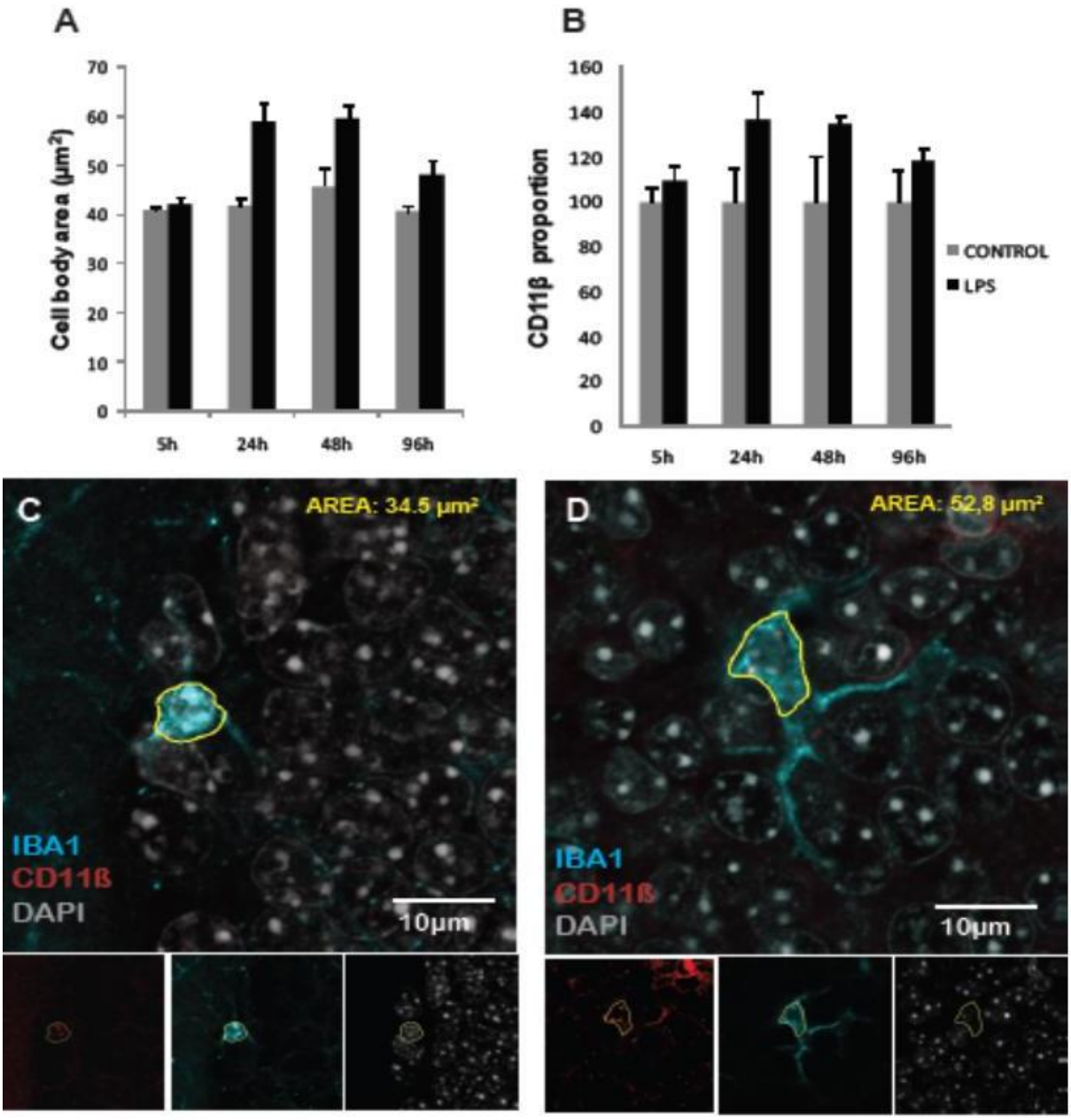

Figure 3-3. LPS-injected animals show an active microglial phenotype. A) Average cell body area reveals hypertrophy at 24h and 48h and no significant difference at 5h and 96h (vertical axis: cell body area expressed in µm2; horizontal axis: time). B) Average proportion of CD11β positive cells in the short term after LPS-saline administration showing a overall higher expression of CD11β in LPS treated mice (vertical axis: variation from control groups proportion of $CD11\beta^{+}$ microglia). C) Mouse killed 24h after saline control solution administration showing a cell body area of 34.5µm2 (perimeter selected in yellow) and D) mouse killed 24h after LPS injection showing an area of 52.8 µm2. Microglial cells identified with IBA-1 (light blue: IBA-1) and the presence of nucleus (gray: DAPI), CD11β activation marker red (red: CD11β)

Intraperitoneal LPS Increases Microglia CD11β Expression.

To test weather peripherally administered LPS stimulated microglia assumed an active phenotype we quantified the proportion of IBA-1^{+} microglia that expressed the marker CD11β. An ANOVA exploring the effects of treatment (Control and LPS) and/or time between LPS administration and death (5h, 24h 48h and 96h) found a statistically significant effect of treatment ($F(1,16)=8.98$; $p<0.01$) but not time effect ($F(3,16)=2.74$; $p=0.077$) or treatment x time interaction effect ($F(3,16)=0.37$; $p=0.77$). Newman Keuls post-hoc analysis confirmed higher expression of CD11β was found in LPS-treated animals versus saline-treated animals ($p<0.01$) as shown in Figure 3-3.

Peripheral LPS Does Not Stimulate an Active Microglial Morphology in the Long Term

We quantified microglia soma areas to determine whether microglia exhibited activated but not phagocytic morphologies at 1 week and 4 weeks after peripheral LPS. An ANOVA confirmed that there was neither an effect of treatment ($F_{(1,8)}=1.39$; $p=0.27$) nor time after treatment ($F_{(1,8)}=4.36$; $p=0.07$) and these effects did not interact ($F_{(1,8)}=0.40$; $p=0.54$; see Figure 3-4). This data confirms that microglia did not present with an active phenotype at 1 week nor after up to 1 month after LPS administration.

Intraperitoneal LPS Does Not Chronically Increase Microglial CD11β Expression

The proportion of IBA-1^{+} microglia that expressed the marker CD11β was evaluated in order to test the presence of active non-phagocytic microglia phenotype. An ANOVA exploring the effects of treatment (Control and LPS) and/or time between LPS administration and death (1 week and 4 weeks) found no effect of treatment ($F_{(1,8)}=0.15$; $p=0.70$) or time effect ($F_{(1,8)}=4.17$; $p=0.075$) or treatment x time interaction

effect ($F_{(1,8)}$=0.19; p=0.67).These results show that intraperitoneal LPS does not induce a microglia active phenotype at 1 week and up to a month after LPS administration.

Neuronal Differentiation Is Transiently Compromised by Peripheral LPS

To test neural differentiation rate we evaluated the proportion of new ($BrdU^+$) cells that expressed DCX and/or NeuN, respectively an early progenitor marker and a neuronal marker. An ANOVA exploring the effects of treatment (Control and LPS) and/or time of the first BrdU injection (5h, 1week, 2weeks, and 3 weeks) and/or phenotype (DCX, DCX/NeuN, and NeuN) found an effect of phenotype (F(2,32)=319; p<0.01), but no statistically significant effect of treatment (F(1,16)=3.94; p=0.06) or time (F(3,16)=1.99; p=0.15). However, a combined effect of treatment x time (F(3,16)=5.12; p<0.05) was found.

As expected, no effect was revealed within control groups (p>0.78). LPS-treated mice that received BrdU 5h after LPS versus saline-treated revealed a statistically significant difference (p<0.05), and no difference between LPS-treated and saline-treated animals was found at 1, 2 or 3 week (p>0.60). This result indicates that neurogenesis is down regulated in animals injected 5h after LPS with BrdU but intraperitoneal LPS does not affect neurogenesis at 1 week or after up to 1 month. Specifically the variation in mice injected with BrdU 5h after LPS was found within new cells expressing BrdU/DCX/NeuN as shown in Figure 3-5 and no statistically significant difference was evaluated considering cells expressing BrdU/DCX or BrdU/NeuN. Furthermore independently from treatment the majority of new cells expressed BrdU/DCX/NeuN and lower percentage of cells expressing BrdU/DCX or BrdU /NeuN were found.

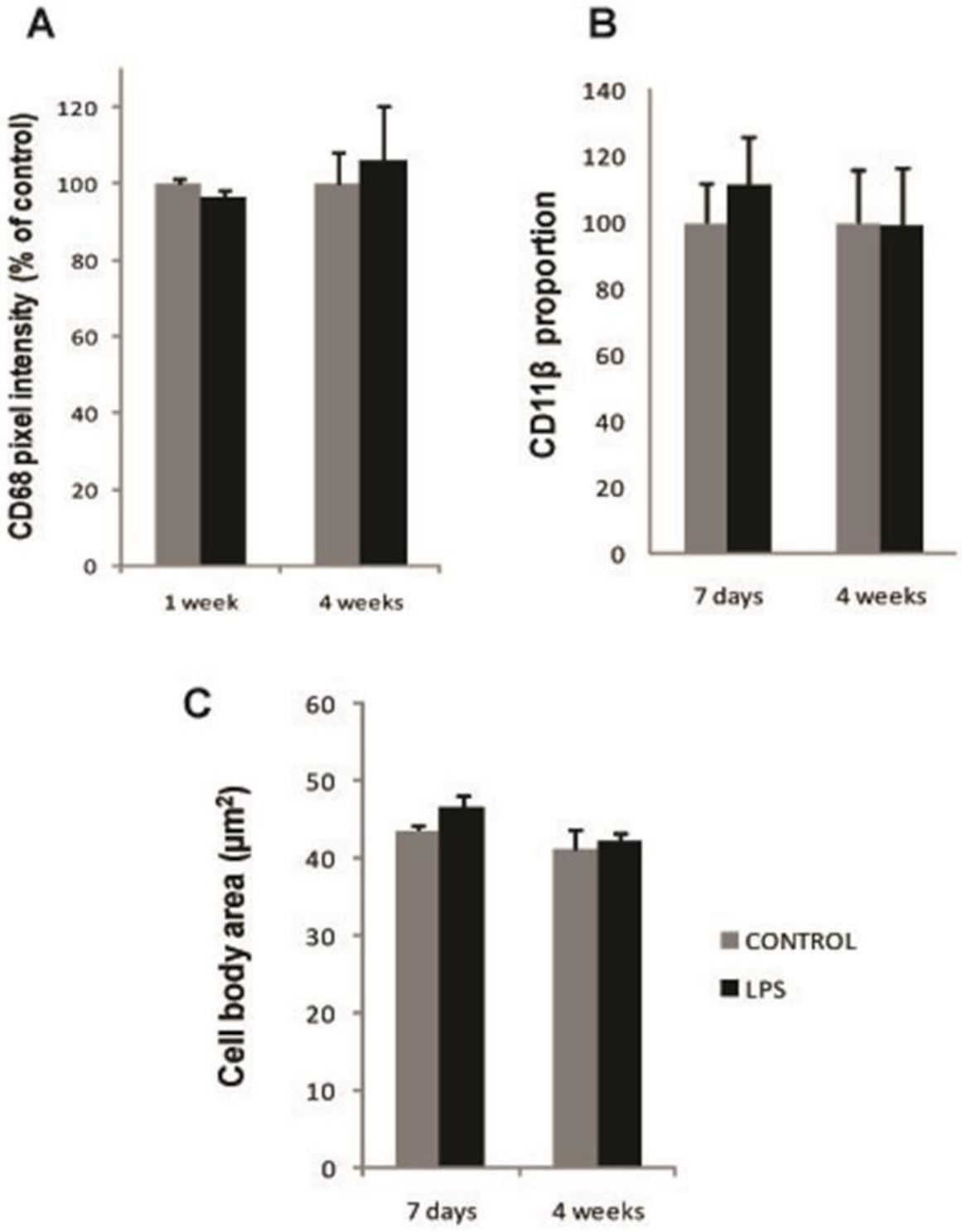

Figure 3-4. Microglia don't show activation at 1 and 4 weeks. (A) Expression of CD68 evaluated with pixel intensity revealing no effect of LPS. (B) Proportion of CD11β positive cells express as variation from control. (C) Cell body area expressed in μm^2

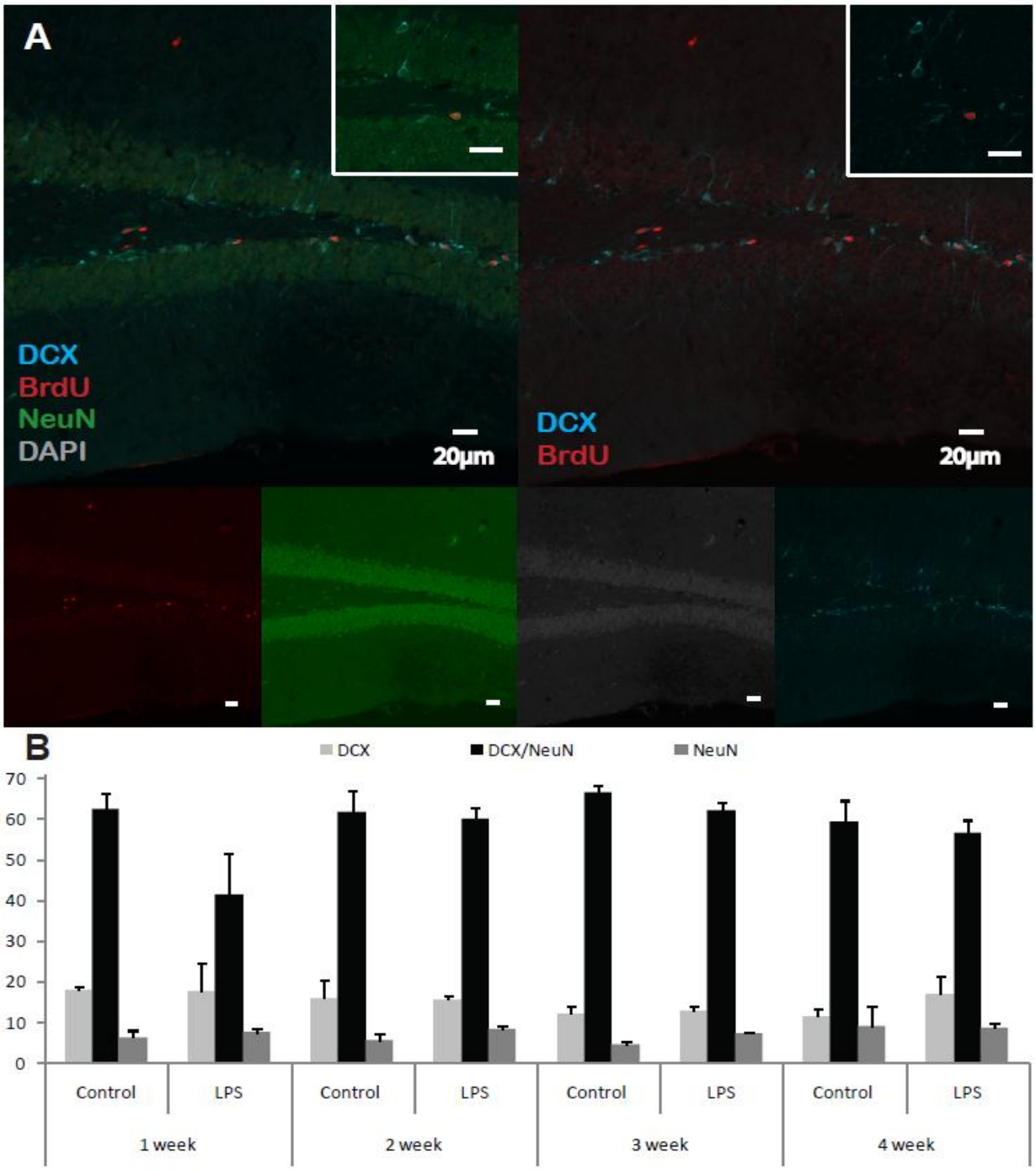

Figure 3-5. Analysis of progenitors phenotype revealed a reduction in BrdU/DCX/NeuN proportion of new cells in animals treated with BrdU 5h after LPS administration. No difference was revealed at 1 week, 2 weeks or 3 weeks after LPS-saline injection. (A)Animal injected with BrdU one week after LPS-saline. A BrdU/DCX/NeuN^{+} cell is shown at higher magnification.(B) Phenotype expression (horizontal axis: time; vertical axes: percentage referred to total BrdU positive cells).

CHAPTER 4
DISCUSSION

Understanding the longevity of the effects of a systemic infection on hippocampal neurogenesis is important because new neuron number has been linked to memory (Deng et al. 2010, Speisman et al. in press). In the current experiment, we found that peripheral injection of bacterial LPS decreased neurogenesis in the week after its administration, but not in the weeks after. Microglia were activated by LPS in the days after administration but not in the following weeks. Microglia activation consisted of CD11β reactivity and the appropriate morphological phenotype, suggesting the presence of inflammatory cytokines. Interestingly, CD68 reactivity was not expressed after stimulation with LPS, suggesting that the microglia did not become phagocytic. Therefore, a single LPS injection only affects hippocampal neurogenesis in the week after its administration. However, approximately 10,000 new granule neurons are added to the hippocampus each day (Cameron & McKay 2001) and LPS-induced disruption of this phenomenon in the week after LPS may ultimately produce profound effects on hippocampus-dependent learning and memory.

The finding that a peripheral injection of LPS decreases neurogenesis in the days after its administration is consistent with previous reports of the effects of both systemic and peripheral LPS injection, including work done previously in our lab (Asokan & Ormerod 2009, Monje et al. 2003, Ekdahl et al. 2003). Monje and colleagues (Monje et al. 2003) labeled cells with BrdU beginning the day of LPS injection and on each of the 5 days afterward. Multiple BrdU injections are used to mark a significant number of dividing cells but cannot detect the precise days an effect occurs on. Taking this into account LPS- and saline-treated animals killed at 1 week have similar numbers of new

cells but a 35% reduction the proportion of new cells that acquire a neuronal phenotype. Monje and colleagues (2003) showed that the number of $CD68^+$ activated microglia correlated negatively with the number of new neurons. In corresponding *in vitro* experiments they showed that LPS-activated microglial conditioned media, IL-6 and TNF-α could ablate neurogenesis. The role of microglia in reducing hippocampal neurogenesis was confirmed by showing that NSAIDs could reduce microglial activation and protect neurogenesis from the effect of LPS (Monje et al. 2003).

Another important study confirming that LPS reduces neurogenesis by stimulating a neuroinflammatory response was published by Ekdahl and colleagues (Ekdahl et al. 2003). They focused on intracranial administration of LPS applied for 28 days at the end of which BrdU was administered. Similar to Monje and colleagues' work (2003), Ekdahl and colleagues showed that after a 2 week survival period similar numbers of new cells could be detected in the dentate gyri of LPS- and saline-treated mice, but there was a 85% reduction in the number of neurons produced in LPS-treated mice. The effect of centrally injected LPS on activated microglia and hippocampal neurogenesis could be prevented by minocycline, linking microglial activation to the effects of central LPS.

In our study, injected BrdU (which is active for ~4h) 5h after injecting LPS and examined the phenotype of the $BrdU^+$ cells after 1 week. Consistent with published work, we found a decreased proportion of new cells that adopted a neuronal phenotype. Our data also suggest that dividing or newly divided $BrdU^+$ cells are affected by the neuroimmune cascade produced by peripheral LPS shortly after their division and in fact, preliminary work from our laboratory suggests that the number of Sox2/$Prox\text{-}1^+$ type 2a cells that emerge within ~1d after BrdU are decreased by LPS. This result is

particularly important because it reveals that already in the early development of neural progenitors LPS intraperitoneal down regulates differentiation.

Broad spectrum NSAID (i.e.minocycline) have been applied in order to control the inflammation and have shown good results in restoration of neurogenesis (Monje et al. 2003, Ekdahl et al. 2003). These drugs have an effect on COX-1 and COX-2 and have shown to reduce microglia cytokine production as well as they might be reduce BBB permeability after LPS administration and prevent a possible afflux to the CNS of peripheral monocytes (Aid *et al.* 2010). On the other end these broad spectrum NSAID are associated with several adverse effects that could be avoided with more selective drugs. An interesting example is given by PPARγ-selective NSAID that have shown to prevent inflammatory induced reduction in neurogenesis and furthermore have shown to prevent changes in hippocampal related cognitive functions (Ormerod et al. under review).

We attempted to elaborate on how LPS activates microglia to ablate neurogenesis using morphological measures. LPS induced hypertrophy in IBA-1^{+} cells within 24h that resolved by 96h. We also detected an overall, time-independent LPS-induced increase in the expression of the activation marker CD11β on IBA-1^{+}ve cells. These findings suggest that a non-phagocytic phenotype emerges shortly after LPS administration. We and others have found that LPS stimulates a robust hippocampal cytokine response within a few hours of its administration (Asokan & Ormerod 2009, Banks & Erickson 2010). Thus, while the elaboration of cytokines may be correlated with CD11β expression, microglial morphological changes appear much later. This might be due to the time delay necessary to express and organize cytoskeleton proteins and produce a

macroscopic morphological change in the cell body detectable with the technique applied, based on confocal microscopy and manual delineation of cell body perimeter. This morphology is more consistent with an activated non-phagocytic phenotype, which is interesting because LPS does not appear to induce cell death among new cells.

Monje and colleagues (2003) detected an increase in CD68 (ED1) expression among IBA-1^{+} cells following peripheral LPS injection in rats, which differs from our finding that CD68 expression was similar on microglia in the hippocampi of LPS- and saline-treated mice. A possible explanation for the differences between studies is that LPS may affect the neuroimmune response of rats differently than mice. Indeed higher doses are required to observe similar effects of LPS on hippocampal neurogenesis in mice versus rats. Ekdahl and colleagues (2003) show a higher CD68 expression in murine microglia that might indicate phagocytic activity. However, we detected no differences in the survival of new cells that would indicate higher or lower incidences of phagocytosis. In this case, the difference might also be due to LPS being delivered intracerebrally by Ekdahl and colleagues, but intraperitoneally in the current study. A confirmation to this hypothesis can be found in Schwartz group researches that revealed a different effect of microglia when their activation is engendered by a controlled mediation of peripheral T-cells (Butovsky et al. 2006). A much simpler explanation is that we used much higher antibody dilutions which may have improved the detection sensitivity of the CD68 assay. Nonetheless, future work that stimulates phagocytic and non-phagocytic activated microglial phenotypes may shed light upon how neuroimmune signaling impacts hippocampal neurogenesis.

In our long term experiments focusing on a time period going from 1 week up to a month we revealed no microglia activation. The absence of activation apparently contrast other studies on rodens, in particular Monje and colleagues (2003) revealed an increase of ED1(CD68) expression at 1 week due to LPS in rats. In this case, a possible explanation could be due to a faster extinction of the inflammatory response in mice, as we used, versus rats. Our findings suggest the absence of a phagocytic activity following LPS inflammation at all time points examined. This might indicate the absence of cellular death (i.e. neuronal death) and subsequently no need to engulf cellular fragments. Furthermore no effect on neurogenesis has been revealed after 1 week indicating that the neurogenic niche has reestablished his original homeostasis. Particularly, microglia resting phenotype expressed at 1 week and after appears to give the same indication of a restored equilibrium in the neurogenic niche, and at the same time, resting microglia might contribute in preserving the pro-neurogenic environment in the hippocampus in the long term after LPS.

Future developments consist of the assessment of particular cytokines responsible for the reduction in neurogenesis within a subset of 35 possible candidates through Bio-Plex technology, work that is already in progress in our laboratory. Particularly, the effect of specific cytokine(s) identified will be tested *in vivo* and *in vitro*. A further possible experiment will assess what component (i.e. microglia, astrocytes, vasculature cells) of the parenchyma is responsible for the production of the anti-neurogenic molecule(s). To assess this we could harvest different cell types from hippocampi of mice and analyze molecules they produce in response to particular cytokines trough Bio-Plex assays of the culture media. Furthermore this will open the possibility of

targeted *in vitro* study on a specific cell type and co-culture of parenchyma cells in order develop and test different drugs inhibiting the production of previously identified anti-neurogenic molecules.

In conclusion, our findings revealed a reduced neurogenesis during the acute inflammatory phase. A relationship between neurogenesis and memory has been shown (Kempermann & Gage 2002), thus emboldening the importance of preserving neurogenesis in order to avoid negative consequences on hippocampal related spatial memory and behavior (Kempermann & Gage 2002, Ormerod et al. under review). For this reason, the study of mechanisms involved in inflammatory processes that have been shown to impair neurogenesis is of extreme importance. Furthermore, effects similar to the LPS induced inflammation can be commonly experience after an influenza or a viral infection, and the necessity to have a deep knowledge of the processes involved in neuroinflammation will set the basis for research toward a targeted treatment for inflammatory reduced neurogenesis. Such a finding may virtually improve the outcome of many pathological conditions involving neuroinflammation, going from the common flu to neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

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BIOGRAPHICAL SKETCH

Alessio Tovaglieri was born in Busto Arsizio, Italy in 1988. He obtained his Bachelor of Science in biomedical engineering in 2010 from Politecnico di Milano, Italy while developing his undergraduate thesis project at Université Libre de Bruxells, Belgium with support from an Erasmus grant. He continued his postgraduate study at Politecnico di Milano focusing on his work on cell and tissue engineering and biotechnology in biomedical engineering.

As a recipient of the Atlantis CRISP dual degree grant he first took classes at University of Strasbourg, Strasbourg, France and then moved to the University of Florida to join the J. Crayton Pruitt Family Department of Biomedical Engineering, Florida, United States of America to complete his Master of Science in biomedical engineering under the supervision of Dr. Brandi Ormerod.

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