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PERIPHERAL LIPOPOLYSACCHARIDE CHALLENGE TRANSIENTLY REDUCES HIPPOCAMPAL NEUROGENESIS AND ACTIVATES MICROGLIA

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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
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

SUMMARY

Neurogenesis is a process that continues throughout all life (Altman & Das 1965, Lois & Alvarez-Buylla 1994, Cameron *et al.* 1993). Adult 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 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 (Squire 1992, Aimone *et al.* 2011, Deng *et al.* 2010). Furthermore neural progenitors have been harvested from different areas of the brain and they still show potential to differentiate *in vitro* in any brain cell type. This capability might be exploited in order to improve the intrinsic capacity of the brain to regenerate and might be the base of future stem cell based therapies.

Adult neurogenesis has shown to play an important role in maintaining functional and efficient neural connections in the hippocampus and olfactory bulb. In order to analyze neural progenitor proliferation and differentiation *in vivo* a timidyne analog bromodeurixidyne (BrdU) molecule can be used. BrdU once injected remains in circulation for few hours and during this time lapse it gets incorporated in dividing cells during the synthesis phase of mitosis. Animals can be then killed hours or days after BrdU administration and tissue can be harvested and trough immunohistochemical procedures new cells identified. Thanks to these procedures several stages of neural progenitors evolution have been identified, based on proteins expression and morphology. In particular at one week new hippocampal cells that have started differentiation into neurons express the early neural marker doublecortin (DCX) and neural nuclei (NeuN).

Many intrinsic, extrinsic and epigenetic factors have been identified and they 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). We focused in particular in neuroinflammation in order to quantify reduction in neurogenesis associated with this process and to identify possible causes and treatments. In particular previous study from Ormerod lab as well as other independent studies revealed a reduction of neurogenesis due to neuroinflammation (Ekdahl *et al.* 2003, Monje *et al.* 2003, Asokan & Ormerod 2009).

In all these studies in order to induce an inflammatory response they relied on the use of lipopolysaccharide (LPS). 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). 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).

A cytokines analysis in the hippocampus and in blood serum revealed a rapid increase of pro-inflammatory cytokines within the first 4 days. In particular we focused our interest on microglia since they have a high cytokines production capability once stimulated and they might play an important role during the inflammatory process. 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.

Among microglia, 4 activation states or phenotypes can be readily distinguished using modern imaging and phenotyping techniques (Streit & Xue 2009). 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. Activated non-phagocytic microglia become more highly branched and hypertrophic and produce 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. Finally, a dystrophic phenotype is exhibited by microglia in the aging CNS or after chronic over-activation (Streit & Xue 2009). These 4 phenotypes are currently considered the standard for classifying microglia phenotypically.

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). Several markers can be used in order to detect microglial activation. Cluster of Differentiation 68 (CD68) is expressed at low-levels on all monocytes/macrophages and microglia, as well as other cell types but its expression increases with phagocytosis (Xue et al. 2010, Micklem *et al.* 1989). 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. Furthermore since hypertrophy of the cell body is associated with microglia activation we measured soma area.

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 *in vivo* manipulations that induce neuroinflammation, such as LPS administration. Cultured NPCs exposed to LPS-stimulated microglia produce new cells that are significantly less likely to acquire a neuronal phenotype. Based upon published

reports, activated microglia remain key suspects of the production of pro-inflammatory cytokines.

On the other hand, activated microglia appear to protect mature neurons. In fact, resting and even activated microglia 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 fate and may also 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 pyramidal neurons in the cornu ammonis of the hippocampus (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. For these reasons we decided to test the effect of LPS induced inflammation on microglia and neurogenesis in the short and long term. In this thesis our work will be presented divided in two experiments; the first analyzing short term effects of LPS on microglia and the second experiment accessing microglia and NPCs phenotype in the long term (see figure 0-1).

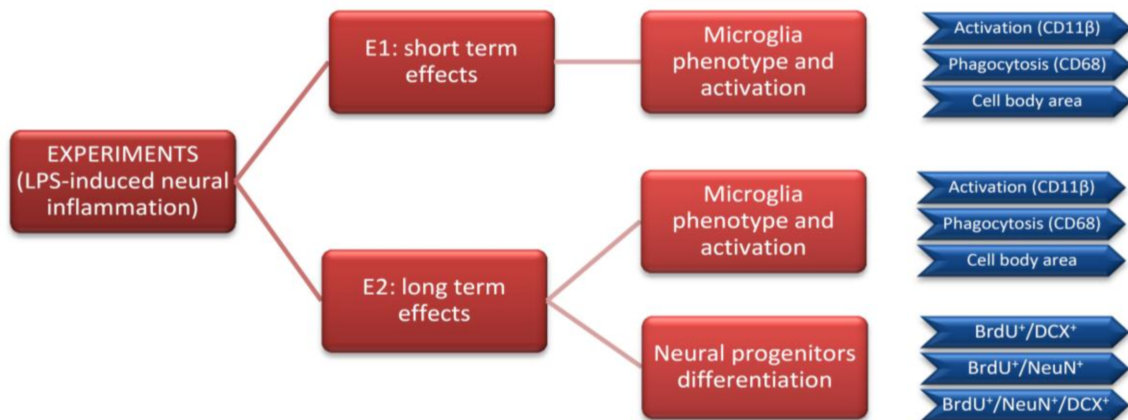

Figure 0-1. Diagram representing a scheme of the two experiments developed *in vivo*. In the first experiment (E1) we accessed the effects of LPS, injected intraperitoneally, on microglia activation and phenotype within the first 4 days. In particular we evaluated microglia activation and phagocytosis considering the expression of respectively CD11β and CD68. In the second experiment (E2) we evaluated microglial and neural phenotype in a period from 1 to 4 weeks after LPS administration. To access neural phenotype we measured the proportion of BrdU+, BrdU+/DCX+, BrdU+/DCX+/NeuN+, BrdU+/NeuN+ cells.

The first experiment was performed in order to evaluate the effects of neuroinflammation on microglia within the first 4 days after intraperitoneal LPS administration. One week after arrival half of the animals were treated with lipopolysaccharide (5mg/kg) to induce transient illness and the other half with saline solution (control group) to control for injection stress. Equal numbers of LPS- and saline-treated mice were anaesthetized with ketamine/xylazine either 5h, 24h, 48h or 96h after treatment by transcardial perfusion with saline solution followed by paraformaldehyde. Brains were sectioned and slices were stored in a cryopreservative until stained immunohistochemically. To detect phagocytic microglia, sections were incubated in antibodies that allow the detection of microglia (anti-IBA-1; Imai et al. 1996), and antibodies indicating the phagocytic activity (rat anti-CD68; Xue et al. 2010, Micklem et al. 1989). To detect activated microglia, sections were incubated in anti-IBA-1 (antibody that labels microglia) and rat anti-CD11β to detect a possible activation state (Smith et al. 1989). The next day, the sections were incubated in the appropriate fluorescent conjugated secondary antibodies. Then confocal images were taken and the proportion of co-expressing IBA-1/CD68 was evaluated in order to quantify phagocytic activity and the proportion of IBA-1/CD11β, over the totality of IBA-1 positive cells, was accessed in order to have an indicator of activation. Then perimeter of cell body area of microglia was selected and area measured based on IBA-1 labelling.

A second experiment was performed in order to access effects of intraperitoneal LPS induced inflammation on neurogenesis and microglia after the first week up to one month. Mice were injected intraperitoneally with LPS (5mg/kg) or an equivalent volume of saline solution (control group). All mice (both LPS and control groups) were injected with BrdU (a marker for newly generated cells) and then animals have been killed at 1, 2, 3, and 4 weeks after LPS/saline with transcardial perfusion (for a detailed description of doses and timing see chapter 2). . To confirm the neuronal phenotype of new cells, other sections were incubated in anti-Neuronal Nuclei (NeuN) to detect mature neuronal protein (Cameron et al. 1993, Ormerod *et al.* 2003), anti-doublecortin (DCX) to detect an early stage of neural progenitor differentiation (Chelly 1998) and we used BrdU in order to evaluate newly formed cells (BrdU is a thymidine analog that gets incorporated into cells during DNA synthesis). All sections were incubated in DAPI to reveal cellular nucleus in order to be able to differentiate background extracellular staining from correct cellular

staining. To identify phenotype of new cells, 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.

To detect microglia activation and their phenotype in the long term we injected animals with LPS or saline solution (control) and then killed animals after 1 and 4 weeks after LPS/saline. Then we accessed the proportion of IBA-1^+ cells (IBA-1 is a marker for microglia) coexpressing CD11β (marker indicating microglial activation), CD68 (marker that indicates phagocytic activity) and measure cell body area (hypertrophy of the cell body is index of cellular activation).

Our results from the first experiment accessing short term effects on microglia revealed a constant low expression of CD68 in all microglia. In order to reveal possible group differences (between LPS and saline treated animals) not visible to the human eye a pixel intensity analysis of co-localized IBA-1/CD68 pixels was performed. No difference between LPS and saline treated groups was detected confirming the absence of phagocytic activity in the short term. A statistically significant overall higher proportion of Cd11β was found in LPS treated animals relative to saline treated animals (control), index of activation. Hypertrophy of the cell body in LPS treated animals was statistically significant at 24h and 48h index of morphological changes due to LPS administration, as shown in figure 0-2. These findings suggest that microglia express an active-non phagocytic phenotype within the first 4 days after LPS administration.

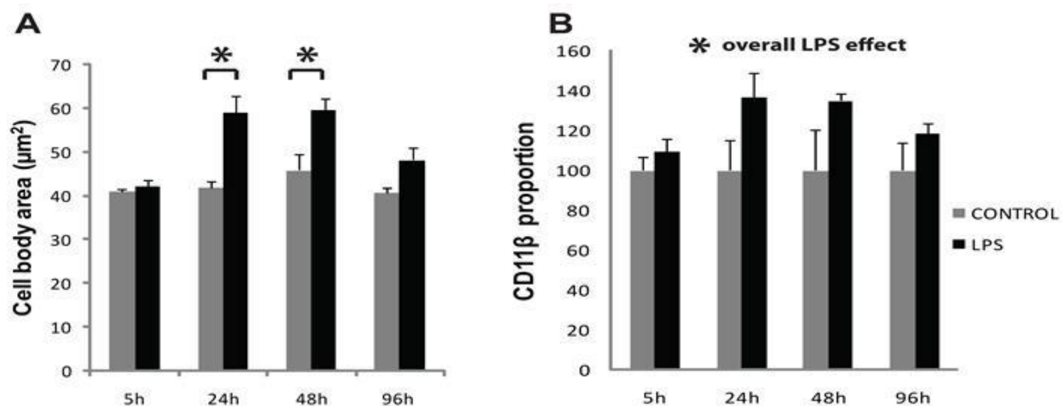

Figure 0-2. LPS-injected animals show an active microglial phenotype. (A) Average cell body area reveals hypertrophy at 24h and 48h and no significant difference at 5h and 96h (vertical axis: cell body area expressed in μm2; horizontal axis: time). (B) Average proportion of CD11β positive cells in the short term after LPS-saline administration showing an overall higher expression of CD11β in LPS treated mice (vertical axis: variation from control groups proportion of $CD11\beta^+$ microglia).

Results in the long term on microglia revealed absence of higher expression of CD11β or CD68 in microglia due to LPS administration and no morphological changes in microglia were detected. This indicates the absence of a long term activation of microglia at least according to the expression of analyzed proteins and morphology. The analysis of new cells revealed a statistically significant effect of LPS in the first week. In particular the proportion of BrdU labeled cells co-expressing DCX and NeuN is significantly reduced by 20%. No reductions in neural differentiation of new cells was found at 2,3 or 4 weeks after LPS intraperitoneal administration (see Figure 0-3) . This reveals that neural differentiation is reduced during the first week after induced inflammation, this effect is not chronic and at 2 weeks already disappears. Analyzing the proportion of cells we can evaluate as in all animals independently from their treatment most of new cells co-express DCX and NeuN index that progenitors have started differentiating into neurons (DCX, early neural marker) but still have not reached complete maturity (NeuN, mature neural marker).

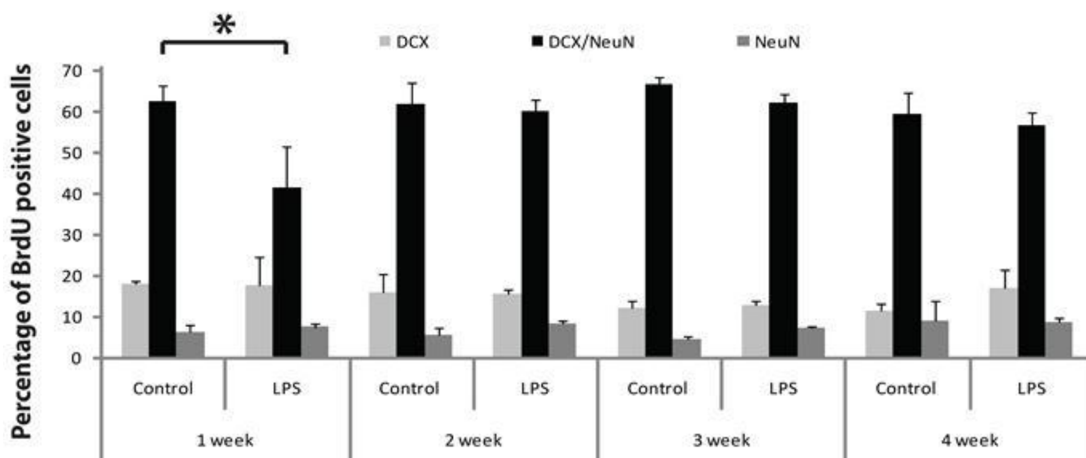

Figure 0-3. Neural progenitors phenotype. As we can see the only statistically significant difference is in the proportion $BrdU^+/DCX^+/NeuN^+$ in animals killed at 1 week and then neurogenesis in restored to its normal rate (horizontal axis: time; vertical axis percentage referred to the total of BrdU positive cells).

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 experiment presented in this thesis, 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, microglia did not appear to have phagocytic activity after LPS administration. 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 administration 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 during a week and found that the total number of newly generated cells (BrdU positive) remained stable but there was a 35% reduction in 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.

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. Similar to Monje and colleagues' work (2003), Ekdahl and colleagues showed an 85% reduction in the number of neurons produced in LPS-treated mice.

In the study described in this thesis we injected BrdU 5h after injecting LPS and examined the phenotype of the $BrdU^+$ cells after 1 week. Consistent with published work, we found a 20% reduction in proportion of new cells that adopted a neuronal phenotype in LPS treated animals. 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. This result is particularly important because it reveals that already in the early development of neural progenitors LPS intraperitoneal down regulates differentiation.

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. Monje and colleagues (2003) detected an increase in CD68 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 in line with the absence of phagocytosis, which otherwise would have been present in case of cellular death. 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.

In our long term experiments focusing on a time period going from 1 week up to a month we revealed no microglia activation. Furthermore no effect on neurogenesis has been revealed after 1 week indicating that the neurogenic niche has reestablished his original homeostasis.

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.

SOMMARIO

La generazione di nuovi neuroni è un processo che prosegue durante l'intera vita (Altman & Das 1965, Lois & Alvarez-Buylla 1994, Cameron et al. 1993). Progenitori neuronali residenti nella zona subventriculare migrano per alcuni centimetri lungo il percorso rostrale prima di differenziarsi in neuroni dopaminergici nello strato periglomerulare e in neuroni GABAnergici nello strato granulare del bulbo olfattorio (Lois & Alvarez-Buylla 1994). Recenti studi suggeriscono che questi nuovi neuroni si integrino nei circuiti responsabili dell'olfatto, dal momento che, nel caso in cui la neurogenesi nel bulbo olfattivo sia ridotta, è stata riscontrata una riduzione nella capacità di discriminare differenti odori (Magavi et al. 2005). Progenitori neurlali generati nella zona sub granulare (subgranular zone; situata tra l'hilus e lo stato granulare) del giro dentato dell'ippocampo migrano nello strato granulare prima di differenziarsi e maturare in neuroni granulari glutaminergici (Palmer et al. 1999, Cameron et al. 1993). Si pensa che questi nuovi neuroni si integrino nel network esistente circa un mese dopo la loro generazione e svolgano un ruolo attivo nell'esecuzione di azioni e comportamenti legati all'attività ippocampale, come ad esempio la navigazione spaziale, dal momento che un ridotto numero di neuroni è stato correlato con ridotti punteggi in test riguardanti apprendimento e memoria spaziali (Squire 1992, Aimone et al. 2011, Deng et al. 2010). Inoltre progenitori neurali sono stati isolati da diverse aree del cervello umano ed è stato dimostrato che queste cellule conservano la capacità di differenziarsi *in vitro* in diversi tipi cellulari presenti nel sistema nervoso centrale (central nervous system; CNS). Questa caratteristica può essere utilizzata per migliorare la capacità intrinseca al cervello di rigenerarsi ed è alla base di numerose ricerche di terapie basate su cellule staminali neurali.

La neurogenesi nell'adulto è un processo fondamentale per il mantenimento di connessioni efficienti e funzionali nell'ippocampo e nel bulbo olfattivo. Per poter analizzare la proliferazione e differenziazione *in vivo* è stato utilizzato in numerosi studi un analogo della base azotata timidina, bromodeurixidina (BrdU). BrdU viene iniettato intraperitonealmente o direttamente nella circolazione venosa dell'animale ed è inglobato dalle cellule nella fase di sintesi del DNA. In questo modo le cellule che si dividono nelle ore successive alla somministrazione di BrdU possono poi essere identificate. Gli animali vengono poi soppressi ore o giorni dopo l'iniezione di BrdU e tramite processi immunoistochimici è possibile identificare le nuove cellule. Grazie a questa procedura è

stato possibile identificare diversi stadi di differenziazione dei progenitori neurali, considerando l'espressione di particolari proteine e la morfologia dei progenitori neurlai. In particolare una settimana dopo la somministrazione di BrdU la maggior parte delle nuove cellule esprime doublecortin (DCX), un marcatore presente in progenitori che hanno iniziato il processo di differenziazione in neuroni e neural nucleai (NeuN), marcatore per cellule neuronali adulte.

Molti fattori, intrinseci ed estrinseci possono influenzare il processo di neurogenesi ippocampale nei mammiferi studiati (Ge et al. 2007). Alcuni di questi fattori, come ormoni e citochine possono attraversare la barriera circolatoria del cervello (blood brain barrier; BBB) dalla circolazione sanguigna e promuovere o inibire la neurogenesi (Ormerod et al. 2004, Czirr & Wyss-Coray 2012). Inoltre segnali generati dalle cellule presenti nella nicchia neurogenica come astrociti, cellule microgliali, cellule endoteliali e altri neuroni possono influenzare la divisione, la maturazione e la sopravvivenza di progenitori neurali (Suzumura et al. 2006). In questo studio ci siamo concentrati in particolare sugli effetti del processo neuroinfiammatorio sulla riduzione della neurogenesi nell'adulto, per identificarne le possibili cause e trattamenti. In particolare precedenti studi presso il laboratorio del Professor Ormerod, così come altri studi effettuati da altri due gruppi di ricerca indipendenti, hanno rivelato una riduzione della neurogenesi direttamente imputabile al processo neuroinfiammatorio (Asokan & Ormerod 2009, Monje et al. 2003, Ekdahl et al. 2003).

In questi studi il processo neuroinfiammatorio è stato generato tramite l'uso di lipopolisaccaride (lipopolysaccharide; LPS). LPS è un endotossina che costituisce la membrana esterna di batteri gram-negativi (Warner et al. 1975). Questa molecola è coinvolta nelle comuni infiammazioni batteriche e può essere trovata nella circolazione sanguigna di pazienti che hanno subito infezioni batteriche (Levin et al. 1970). LPS, quando iniettato intraperitonealmente, causa un aumento della produzione di citochine pro-infiammatorie e induce una condizione infiammatoria nel cervello comparabile con molti tipi di infezioni che possono sopraggiungere in soggetti umani (Turrin et al. 2001).

L'analisi delle citochine ippocampali ha rivelato un rapido aumento di alcune citochine pro-infiammatorie durante i 4 giorni successivi all'infiammazione. In particolare nello studio presentato in questo lavoro di tesi, effettuato presso il Laboratorio di Neural Stem Cell sotto la guida del Professor Ormerod, ci siamo concentrati sull' analisi delle

cellule microgliali dal momento che queste cellule hanno mostrato una spiccata capacità nel produrre citochine in risposta a diversi stimoli, tra cui molecole associate alla risposta infiammatoria. Questo interesse è stato stimolato da recenti ricerche su cellule microgliali attivate da lesioni, invecchiamento (Sheffield & Berman 1998), e patologie neurodegenerative, che sono state associate a deficit cognitivi (Itagaki et al. 1989, Oyebode et al. 1986). L'attivazione delle cellule microgliali è inoltre presente in molte sindromi associate a deficit cognitivi e probabilmente ne costituisce una concausa.

Tra le cellule microgliali è possibile distinguere 4 stati di attivazione e fenotipi utilizzando le moderne tecniche di imaging e identificazione del fenotipo (Streit & Xue 2009). Cellule microgliali possono essere a riposo, attivate non fagocitiche, attivate fagocitiche e distrofiche. Cellule microgliali sono presenti nel sistema nervoso centrale in pazienti sani e generalmente esprimono fenotipo chiamato a riposo e contribuiscono attivamente al mantenimento dell'equilibrio nel sistema nervoso centrale e nel mantenimento delle connessioni sinaptiche (vedi Wake et al. 2009). Cellule microgliali nello stato a riposo sono moderatamente ramificate. In caso di infiammazione del sistema nervoso centrale non associata con una rilevante morte cellulare, le cellule microgliali assumono un fenotipo attivo-non fagocitico. Cellule microgliali attive non fagocitiche hanno una maggiore ramificazione dei processi cellulari e presentano ipertrofia del corpo cellulare associata ad una produzione di citochine pro- e anti-infiammatorie, ritenute necessarie per il ripristino dell'omeostasi nel sistema nervoso centrale. L'attivazione fagocitica si pensa venga indotta da una rilevante morte cellulare o da un danneggiamento della barriera circolatoria del cervello; questo fenotipo è caratterizzato da una ridotta ramificazione e ipertrofia del corpo cellulare. Infine, un fenotipo distrofico è espresso da cellule microgliali nel sistema nervoso centrale di pazienti in età avanzata o in seguito ad una eccessivamente prolungata attivazione (Streit & Xue 2009). Questi 4 fenotipi sono comunemente considerati come standard per la classificazione di cellule microgliali.

Oltre all'esame dello stato di attivazione basato sulla morfologia delle cellule microgliali in diversi contesti sperimentali, la loro attivazione può essere riscontrata tramite l'analisi di bio-marcatori proteici. La molecola di legame al calcio ionizzato (Ionized calcium binding adaptor molecule-1; IBA-1) è una proteina espressa nel corpo cellulare e nei processi dei monociti/macrofagi presenti nella circolazione sanguigna e nella controparte del sistema nervoso centrale costituita dalle cellule microgliali (Xue et al.

2010, Imai et al. 1996). Numerosi marcatori possono essere utilizzati per individuare lo stato di attivazione delle cellule microgliali. La molecola del gruppo di differenziazione 68 (cluster of differentiation 68; CD68) è lievemente espressa in tutti i monociti/macrofagi e microglia, così come in altri tipi di cellule, ma è da sottolineare come la sua espressione aumenti in caso di attività fagocitica (Xue et al. 2010, Micklem et al. 1989). La molecola del gruppo di differenziazione 11β (CD11β) è espressa in monociti/macrofagi e cellule microgliali attive fagocitiche e attive non fagocitiche (Zhang & Plow 1999, Smith et al. 1989). Nel corso di questo studio abbiamo utilizzato entrambi i marcatori in modo da stabilire il fenotipo delle cellule microgliali. Abbiamo inoltre effettuato misure sistematiche dell'area del corpo cellulare di microglia dal momento che ad un attivazione cellulare è associata ipertrofia.

Il ruolo neuroprotettivo o neurotossico delle cellule microgliali è tuttora al centro di un vivo dibattito. Cellule microgliali attivate sembrano ridurre la produzione di nuovi neuroni (Monje et al. 2003). Nell'ippocampo di roditori adulti, il numero di nuovi neuroni prodotti è stato messo in correlazione *in vivo* con il numero di cellule microgliali attivate successivamente a somministrazione di LPS. E' stato inoltre mostrato come l'aggiunta di cellule microgliali attivate da LPS a culture cellulari di progenitori neurali *in vitro,* riduca la differenzizzione dei progenitori in neuroni. Considerando le recenti pubblicazioni appare evidente come le cellule microgliali rivestano un ruolo di massima importanza nel processo infiammatorio e nella formazione di nuovi neuroni. Allo stesso tempo, cellule microgliali attivate sembra siano in gradi di proteggere cellule neurali mature. Infatti cellule microgliali sia a riposo che attivate possono facilitare il processo neurogenico nell'ippocampo dell'adulto in diversi modi. Esse possono infatti fagocitare il 50% delle nuove cellule che non raggiunge completa maturazione (Oppenheim 1991). Cellule microgliali possono inoltre regolare direttamente il destino dei progenitori neurali e possono inoltre essere coinvolte nell'eliminazione di frammenti potenzialmente tossici che potrebbero compromettere la maturazione dei neuroni neogenerati come ad esempio un eccesso di glutammato (Streit 2002). Possono anche controllare la sinaptogenesi che avviene tra i neuroni neoformati e i neuroni piramidali nel cornu ammonis dell'ippocampo (Wake et al. 2009, Wake et al. 2012). Dunque, la comprensione ed il controllo delle cellule microgliali durante un'infiammazione che coinvolga il sistema nervoso centrale appare critica nel caso in cui sia necessario mantenere una corretta neurogenesi.

Tenendo inoltre in considerazione che lo scopo di numerose strategie neuroingegneristiche consiste nella sostituzione di parti del network neurale non più funzionali in situazioni patologiche o in seguito a lesioni, appare di cruciale importanza conoscere le molecole neuro-infiammatorie in grado di ridurre la formazione di nuovi neuroni. Per queste ragioni abbiamo deciso di analizzare gli effetti di un'infiammazione indotta da somministrazione di LPS sulle cellule microgliali e sul processo neurogenico a breve e a lungo termine. In seguito questa ricerca verrà presentata divisa in due esperimenti; nel primo esperimento abbiamo analizzato gli effetti a breve termine dell'LPS sulle cellule microgliali e nel secondo esperimento abbiamo valutato gli effetti a lungo termine dell'LPS su cellule microgliali e su progenitori neurali, come illustrato in Figura 0-1.

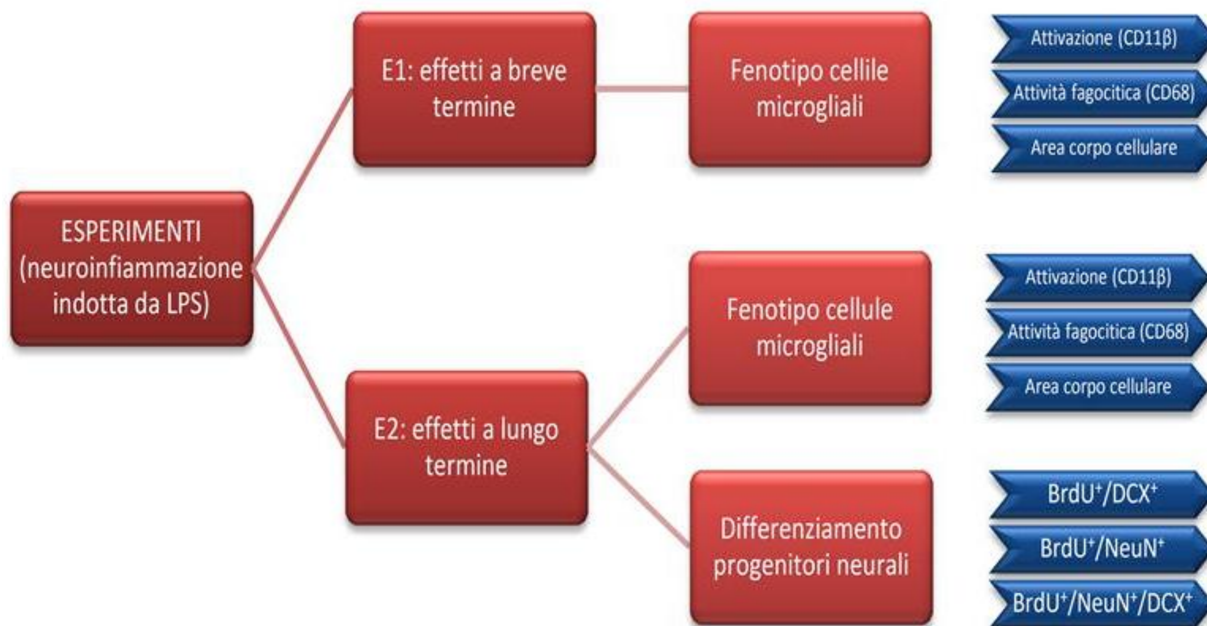

Figura 0-1. In questo diagramma sono rappresentati schematicamente i due esperimenti effettuati *in vivo*. Nel primo esperimento (E1) sono stati analizzati gli effetti sulle cellule microgliali nei primi 4 giorni successivi alla somministrazione intraperitoneale di LPS. In particolare è stata valutata l'attivazione analizzando la proporzione di cellule che coesprimono IBA-1/CD11β, l'attività fagocitica valutando l'espressione di CD68 ed è stata misurata l'area del corpo cellulare. Nel secondo esperimento (E2) è stato valutato il fenotipo delle cellule microgliali e il fenotipo dei progenitori neurali in un intervallo si tempo da 1 a 4 settimane successive alla somministrazione di LPS. Per valutare il fenotipo dei progenitori neurali è stata misurata la proporzione di cellule $BrdU^+$, $BrdU^+/DCX^+$, $BrdU^+/DCX^+/NeuN^+$, $BrdU^+/NeuN^+$.

Il primo esperimento è stato effettuato per valutare gli effetti di un'infiammazione neurale durante i 4 giorni successivi alla somministrazione intraperitoneale di LPS sulle cellule microgliali. Una settimana dopo il loro arrivo metà dei topi sono stati trattati con LPS (5mg/kg) in modo da indurre unprocesso infiammatorio transitorio; l'altra metà degli animali, il gruppo di controllo, è stata iniettata con soluzione salina in modo da tener in conto dello stress indotto dall'iniezione stessa. I topi sono stati successivamente anestetizzati con ketamina/xylazina a 5 ore, 24 ore, 48 ore e 96 ore di distanza e, una volta anestetizzati, sono stati soppressi tramite perfusione trans cardiaca di soluzione salina seguita da paraformaldeide. I cervelli sono stati poi estratti e fissati in paraformaldeide e stabilizzati in saccarosio. I cervelli sono stati sezionati e le sezioni sono state conservate in una soluzione criopreservativa pronte per il processo di colorazione immunoistochimico. Per valutare l'attività fagocitica delle cellule microgliali le sezioni sono state incubate in anticorpi che permettono il riconocimento delle cellule microlgiali (anti-IBA-1; Imai et al. 1996), e anticorpi che inidcano la presenza di attività fagocitica (anti-CD68; Xue et al. 2010, Micklem et al. 1989). Per identificare le cellule microgliali attive le sezioni sono state incubate in un antcorpo specifico per le cellule microgliali (anti-IBA-1) e anticropi che inidcano l'attivazione (fagocitica e non fagocitica) delle cellule microgliali (anti-CD11β; Smith et al. 1989). Il giorno successivo le sezioni sono state incubate in appropriati anticorpi coniugati secondari. Successivamente immagini sono state acquisite tramite microscopia confocale ed è stata valutata la proporzione di cellule che co-esprimono IBA-1/CD68 in modo da quantificare l'attività fagocitica ed è stata analizzata la proporzione di IBA-1^+/CD11β^+ rispetto al totale delle cellule IBA-1^+ in modo da disporre di un indicatore dell'attivazione microgliale.

Un secondo esperimento è stato effettuato in modo da stabilire gli effetti dell'infiammazione indotta sulla neurogenesi e sulle cellule microgliali nell'intervallo di tempo tra una settimana e un mese dopo l'iniezione di LPS (5 mg/kg). Per valutare la neurogenesi a lungo termine ai topi è stata somministrata una singola iniezione di LPS o un volume equivalente di soluzione salina. Agli animali (sia gruppi di controllo che LPS) è stato iniettato BrdU (una marcatore per le cellule neogenerate) e successivamente gli animali sono stati sacrificati a 1, 2, 3 e 4 settimane tramite perfusione transcardiaca (per la descrizione dettagliata delle tempistiche e dosi di somministrazione vedi capitolo 2). Per confermare il fenotipo neurale delle cellule neogenerate le sezioni sono state incubate in

anti-Neuronal Nuclei (NeuN), in modo da identificare specifiche proteine neurali (Cameron et al. 1993, Ormerod et al. 2003) e anti-doublecortin (DCX) in modo da identificare progenitori in un primo stadio di differenziazione neuronale (Chelly 1998) ed è stato utilizzato anti-BrdU in modo da poter valutare le cellule negenerate (BrdU è un analogo della timidina e viene inglobato nel DNA delle nuove cellule). Infine tutte le sezioni sono state incubate in DAPI in modo da identificare i nuclei cellulari e poter distinguere in modo chaiaro cellule da eventuali artefatti dovuti all'accumulo di anticorpi in zone extracellulari. Per identificare il fenotipo delle nuove cellule è stata quindi valutata la proporzione di cellule $BrdU^+$, $BrdU^+/DCX^+$, $BrdU^+/DCX^+/NeuN^+$, $BrdU^+/NeuN^+$ in modo da avere un indicatore della differenziazione dei progenitori neurali.

Per valutare l'attivazione delle cellule microgliali ed il loro fenotipo a lungo termine è stato iniettato LPS o soluzione salina (gruppo di controllo) e successivamente i topi sono stati sacrificati 1 e 4 settimane dopo la somministrazione di LPS/soluzione salina.
Abbiamo quindi esaminato la proporzione di cellule $IBA\text{-}1^+$ (marcatore per cellule microgliali) che co-esprimono CD11β (marcatore che rivela l'attivazione delle cellule micrgliali), CD68 (marcatore che rivela presenza di attività fagocitica) e misurato l'area del corpo cellulare (l'ipertrofia costituisce un indice di attivazione cellulare).

I risultati del primo esperimento atto a valutare gli effetti di LPS sulle cellule microgliali a breve termine hanno rivelato una bassa espressione di CD68 in tutte le cellule microgliali, di conseguenza per valutare possibili impercettibili differenze tra i gruppi abbiamo deciso di effettuare un'ulteriore analisi basata sull'intensità dei pixel corrispondenti a CD68 colocalizzati con IBA-1. Anche quest'analisi non ha rivelato alcuna differenza tra i gruppi di animali trattati con LPS e soluzone salina (gruppo di controllo), indice di un'assenza di attività fagocitica delle cellule microgliali nei primi 4 giorni. Complessivamente è stata rilevata una maggiore proporzione di cellule CD11β positive negli animali trattati con LPS, rispetto a quelli trattati con soluzione salina. Ed è stata altresì rilevata ipertrofia del corpo cellulare statisticamente significativa a 24 e 48 ore, indice di cambiamenti morfologici dovuti alla somministrazione di LPS, come mostrato in figura 0-2. Questi risultati suggeriscono che le cellule microgliali esprimono un fenotipo attivo non fagocitico nei primi 4 giorni successivi alla somministrazione intraperitoneale di LPS.

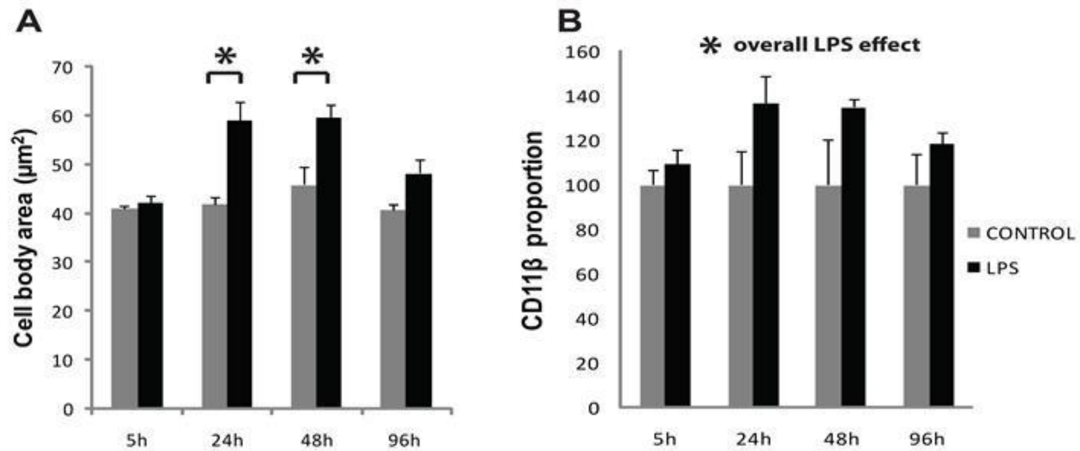

Figure 0-2. Animali iniettati con LPS presentano cellule microgliali attivate. (A) Media dell'area del corpo cellular rivela ipertrofia statisticamente significativa a 24 e 48 ore e nessuna differenza statisticamente significativa a 5ore e 96 ore successivamente alla somministrazione di LPS (asse verticale: area del corpo cellulare espressa in μm^2; asse orizzontale: tempo). (B) Proporzione di cellule microgliali $CD11\beta^+$ a breve termine rivela una espressione maggiore di CD11β considerando compessivamente i topi trattati con LPS rispetto ai gruppi di controllo trattati con soluzione salina (asse verticale: variazione percentuale dai gruppi di controllo).

I risultati del secondo esperimento atto a valutare gli effetti a lungo termine sulle cellule microgliali non ha rivelato differenze nella espressione di CD11β o CD68 nelle cellule microgliali dovuta alla somministrazione di LPS. Ciò indica l'assenza di un'attivazione a lungo termine delle cellule microgliali basandosi sull'espressione delle proteine analizzate e sulla loro morfologia. L'analisi delle cellule neogenerate ha rivelato un effetto statisticamente significativo durante la prima settimana seguente la somministrazione di LPS. In particolare la proporzione di cellule che coesprimono DCX e NeuN è ridotta del 20% in seguito alla somministrazione di LPS (vedi figura 0-3). Nessuna riduzione nel processo neurogenico è stata rivelata a due, tre o quattro settimane. Ciò indica che la riduzione della neurogenesi avviene durante la prima settimana e questo effetto non è cronico e nella seconda settimana non è già più rilevabile. L'analisi della proporzione di diversi marcatori indipendentemente dal trattamento subito dall'animale ha rivelato come la maggior parte dei progenitori neurali esprima DCX e NeuN, indice del fatto che i progenitori dopo una settimana dalla loro generazione hanno iniziato a differenziarsi in neuroni (DCX, marcatori neurale della prima fase di differenzazione) ma non hanno ancora raggiunto completa maturità (NeuN, marcatori per neuroni giunti a completa maturazione).

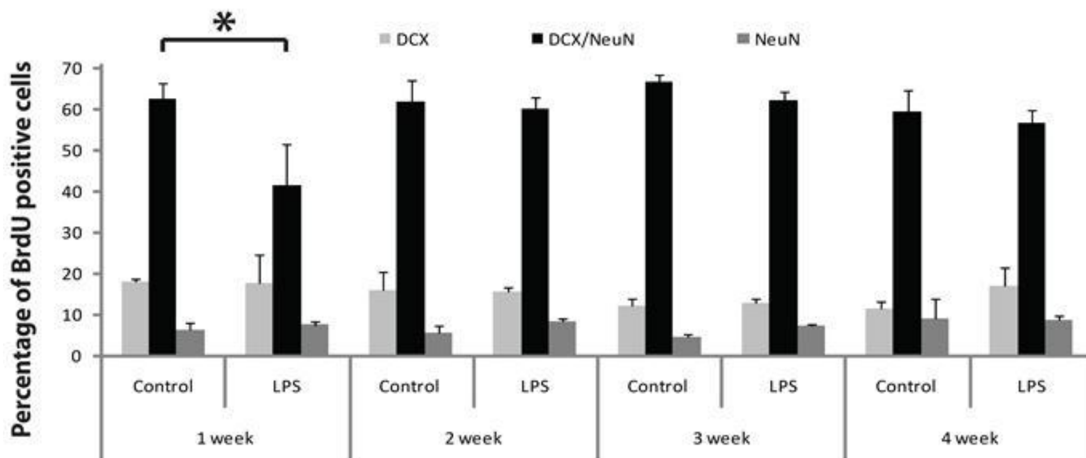

Figura 0-3. Fenotipo dei progenitori neurali. Come possiamo verificare dal grafico l'unica differenza statisticamenete significativa è presente nelle cellule che esprimono $BrdU^+/DCX^+/NeuN^+$ negli animali sacrificati ad una settimana e successivamente non vi sono più differenze tra i gruppi di controllo e LPS. Quindi dopo un'iniziale riduzione, la neurogenesi ha riassunto valori normali (asse orizzontale: tempo; asse vertical percentuale riferita al numero totale di cellule $BrdU^+$).

La comprensione della durata degli effetti di un'infiammazione sistemica sulla neurogenesi ippocampale è di estrema importanza dal momento che una riduzione della neurogenesi ippocampale è stata correlata a deficit della memoria spaziale (Deng et al. 2010, Speisman et al. in press). Negli esperimenti presentati in questa tesi abbiamo riscontrato come l'iniezione intraperitoneale di LPS riduca la neurogenesi nella settimana successiva alla somministrazione, e questo effetto non è più riscontrabile nelle settimane successive. Le cellule microgliali presentano attivazione nei giorni successivi alla somministrazione di LPS ma non nelle settimane successive. L'attivazione delle cellule microgliali consiste in una maggiore espressione di CD11β accompagnata da un appropriato fenotipo morfologico che suggerisce la presenza e possibile produzione di citochine infiammatorie. È inoltre interessante il fatto che non sia stata rilevata attività fagocitica negli animali trattati con LPS. Dunque una singola iniezione di LPS influenza la neurogenesi ippocampale esclusivamente nella prima settimana successiva alla somministrazione di LPS. Ciononostante circa 10.000 nuovi neuroni granulari si aggiungono ogni giorno allo strato granulare dell'ippocampo (Cameron & McKay 2001) e la distruzione del fenomeno neurogenico indotta da LPS ha la capacità di produrre profondi effetti sull'apprendimento e sulla memoria spaziale, attività profondamente legate all'integrità ippocampale.

Questi risultati riguardanti una riduzione della neurogenesi nella prima settimana successiva alla somministrazione di LPS trovano conferma in altri studi precedenti che hanno riportato l'effetto di iniezioni sistemiche e centrali (intracraniche) di LPS, in studi effettuati nel laboratorio del Professor Ormerod (Asokan & Ormerod 2009, Monje et al. 2003, Ekdahl et al. 2003) e da Ekdhal e colleghi come anche Monje e colleghi. Questi ultimi (Monje et al. 2003) hanno effettuato una marcatura delle cellule in mitosi con BrdU durante la prima settimana e hanno rivelato come il numero totale delle cellule neogenerate (marcate con BrdU) sia rimasto sostanzialmente invariato considerando gli animali trattati con LPS (iniettato intraperitonealmente) rispetto a soluzione salina, ma una proporzione ridotta del 35% di nuove cellule che hanno intrapreso una differenziazione neuronale. Monje e colleghi hanno inoltre mostrato come il numero di cellule microgliali attivate $CD68^{+}$ sia correlato negativamente con il numero di nuovi neuroni. Un altro importante studio in cui è stato confermato l'effetto negativo di LPS sulla neurogenesi è stato pubblicato da Ekdahl e colleghi (Ekdahl et al. 2003). In questo studio si sono concentrati sugli effetti di LPS somministrato tramite un'iniezione intracranica. In maniera simile a Monje anche Ekdahl e il suo gruppo hanno una riduzione dell'85% nel numero di neuroni prodotti.

Nella serie di esperimenti eseguiti nel nostro labortorio e descritti in questa tesi abbiamo iniettato BrdU 5 ore dopo l'iniezione di LPS e abbiamo esaminato il fenotipo delle cellule $BrdU^{+}$ la settimana seguente. In linea con studi precedentemente pubblicati abbiamo riscontrato una proporzione di cellule neogenerate che abbiano assunto fenotipo neurale ridotta del 20% in seguito a somministazione di LPS. I nostri dati suggeriscono che le cellule neogenerate marcate con BrdU siano affette dalla cascata neuro-immune prodotta da LPS iniettato intraperitonealmente già a partire dalle prime ore. Questo risultato è particolarmente interessante dal momento che rivela come già nel primo sviluppo dei progenitori neurali, l'LPS possa ridurre il processo di differenziazione neuronale.

Per valutare gli effetti a breve termine di LPS sull'attivazione delle cellule microgliali abbiamo analizzato l'espressione di IBA-1 colocalizzato con CD11β e CD68 e misurato l'area del corpo cellulare. Monje e colleghi hanno evidenziato un aumento nell'espressione di CD68 tra le cellule cellule $IBA\text{-}1^{+}$ in seguito a iniezione di LPS in ratti; questo risultato è differente da quello riscontrato nei nostri esperimenti in cui non abbiamo rilevato differenza di espressione di CD68 dovuta a somministrazione di LPS. Questa

differenza può essere dovuta alla differenza degli effetti indotti da LPS su topi rispetto a ratti. Effettivamente studi precedenti hanno rivelato come sia necessaria una dose più elevata di LPS nei topi rispetto a ratti per avere una riduzione della neurogenesi. Ekdahl e colleghi hanno mostrato una maggiore espressione di CD68 in cellule microgliali, possibile indice di attività fagocitica. In questo caso la differenza con i nostri risultati può essere attribuita al fatto che LPS è stato somministrato intracranialmente e non intraperitonealmente. Una conferma a questa ipotesi può essere trovata negli studi del gruppo di ricerca di Schwartz che hanno rivelato un effetto differente sulle cellule microgliali nel caso in cui queste vengano attivate in modo controllato da parte delle cellule T situate nella periferia (Butovsky et al. 2006). Una spiegazione più semplice può essere attribuita all'utilizzo di differenti diluizioni degli anticorpi che potrebbe aver modificato la sensibilità di detezione nell'analisi di CD68.

Negli esperimenti effettuati a lungo termine, focalizzati in un periodo che va da una settimana a un mese successivamente alla somministrazione di LPS abbiamo rilevato l'assenza di effetti sull'attivazione delle cellule microgliali. Inoltre non abbiamo riscontrato alcun effetto sulla neurogenesi nel periodo seguente alla prima settimana; ciò indica che probabilmente l'omeostasi della nicchia neurogenica è stata ristabilita.

In conclusione, i nostri risultati hanno rivelato una riduzione della neurogenesi durante la fase acuta del processo infiammatorio. E' stata chiaramente dimostrata in studi precedenti una relazione tra neurogenesi e memoria spaziale (Kempermann & Gage 2002), sottolineando dunque l'importanza di una preservazione della neurogenesi in modo da evitare conseguenze negative sull'apprendimento e sulla memoria spaziali (Kempermann & Gage 2002, Ormerod et al. under review). Per queste ragioni appare di estema importanza lo studio dei processi neuroinfiammatori che possono portare ad una riduzione della memoria spaziale. Inoltre, effetti simili all'infiammazione indotta da LPS possono essere comunemente riscontrati in seguito a un'influenza o infezione virale e una conoscenza approfondita dei processi coinvolti nella neuro infiammazione fornirà le basi per una ricerca di uno specifico trattamento per la riduzione della neurogenesi di origine neuroinfiammatoria. E' auspicabile nonché verosimile che in tempi ravvicinati questi risultati possano migliorare gli esiti del trattamento di numerose patologie che coinvolgono neuroinfiammazione, dalla comune influenza alle patologie neurodegenerative come l'Alzheimer e il Parkinson.

CHAPTER 1:
INTRODUCTION

1.1 Adult Neurogenesis

Neurogenesis in humans and all other mammals studied occurs in the olfactory bulb and hippocampal dentate gyrus throughout life (see Figure 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).

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).

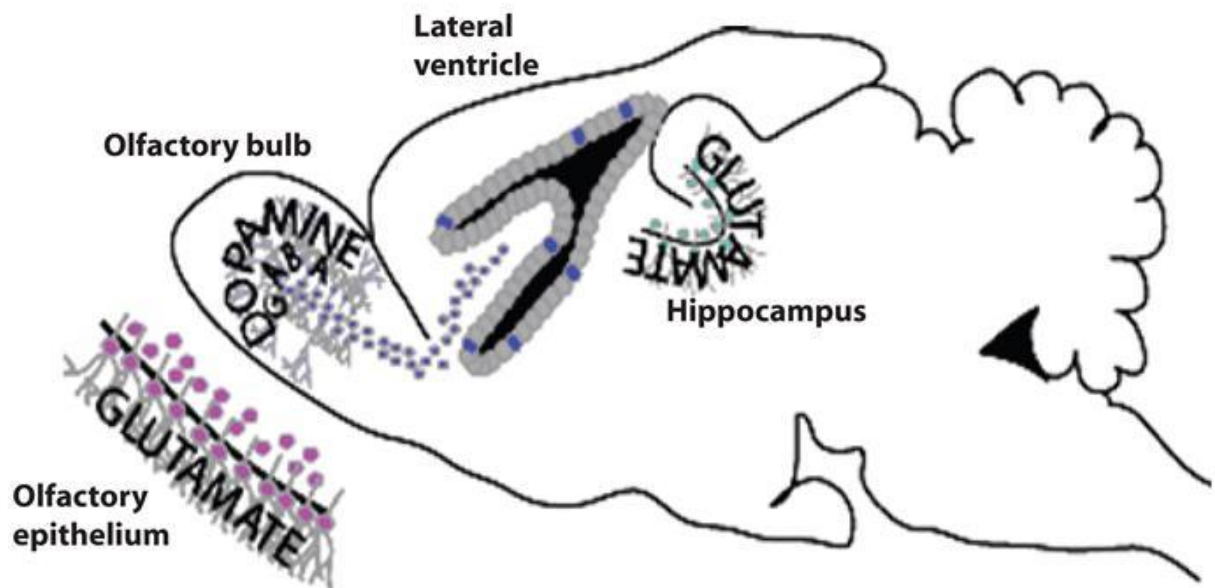

Figure 1-1. Cartoon of a rat brain showing hippocampal and olfactory bulb neurogenesis. NPCs that reside in the subgranular zone between the hilus and granule cell layer divide producing daughter cells. Many of these daughter cells migrate deeper into the granule cell layer and differentiate into granule 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.

1.1.1 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, Ormerod lab 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 environmental enrichment (that consists in an environment that mimics the animal's wild habitat and allows it to express all its natural behaviors) 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 after BrdU injection, the experimenter can evaluate the number of newly generated 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.

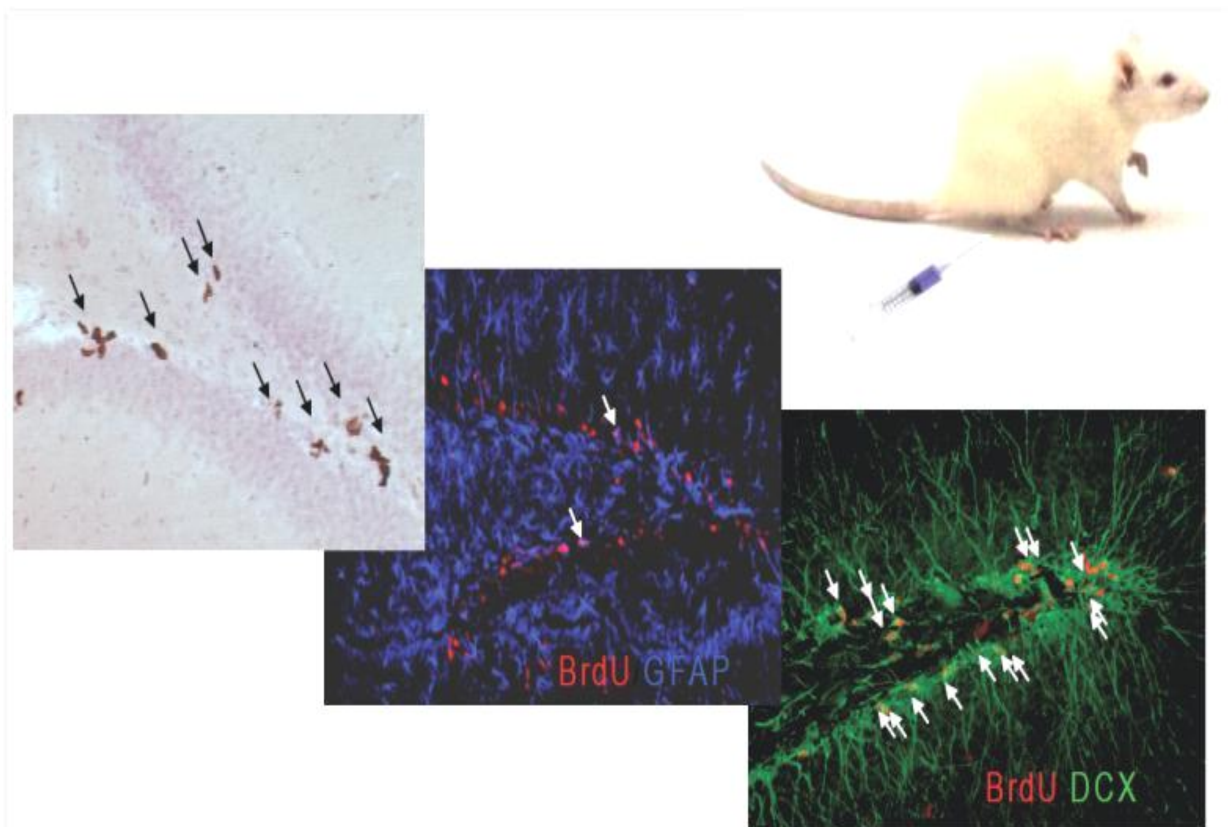

Figure 1-2. Neurogenesis in the dentate gyrus and subventricular zone. Use of Brdu marker injected in mice in order to label dividing cells and examples of himmunohistochemical staining of progenitors expressing the glial marker GFAP or the immature neuronal marker DCX. [Figure courtesy of Dr. Ormerod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

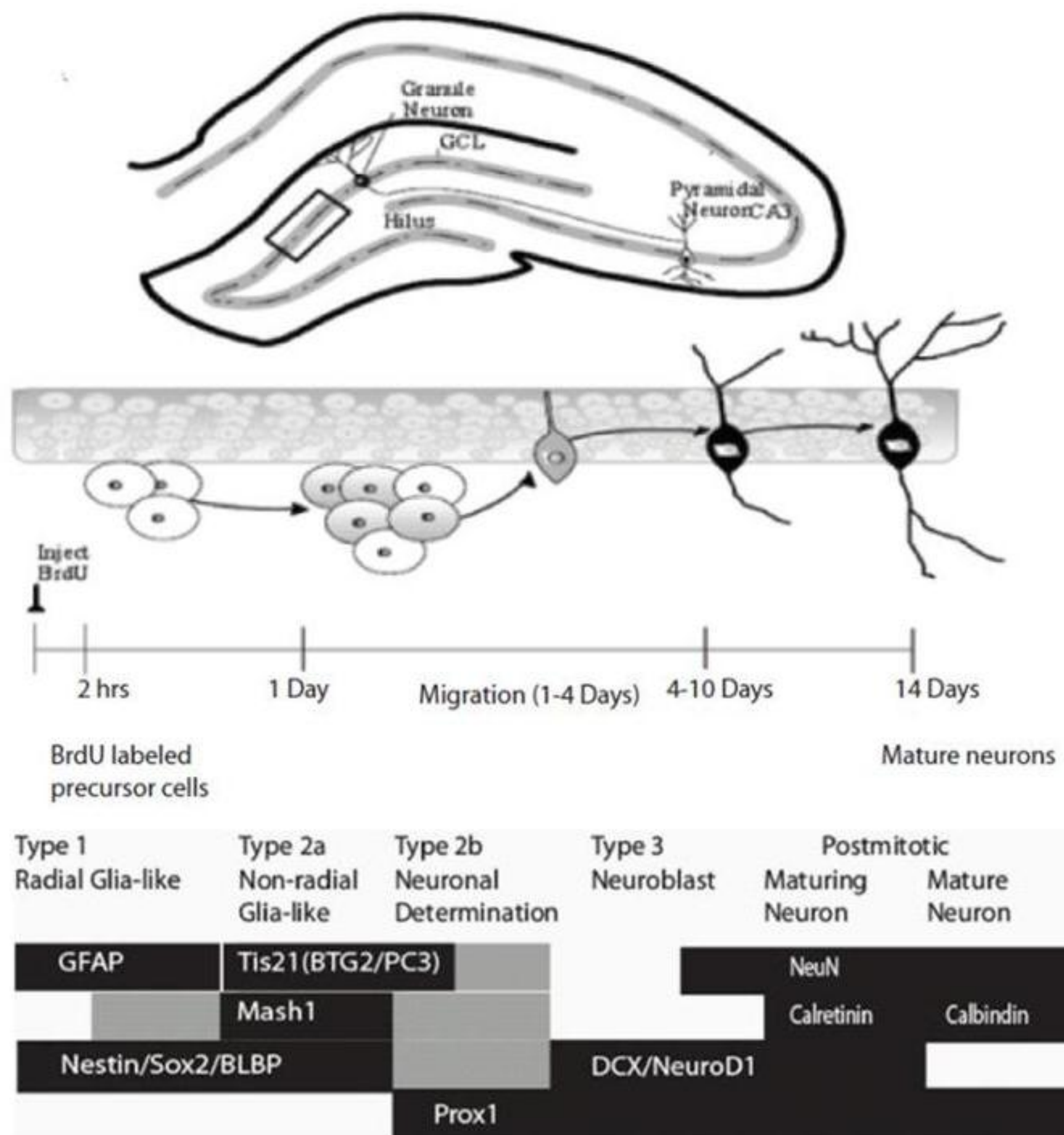

Figure 1-3. Dentate gyrus and different development stages of neural progenitors showing different marker expression associated with the evolutionary stage of neural progenitors. [Figure courtesy of Dr. Ormerod B.K.]

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).

1.2 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).

1.2.1 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. Recent *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 through 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 blood brain barrier 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).

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.

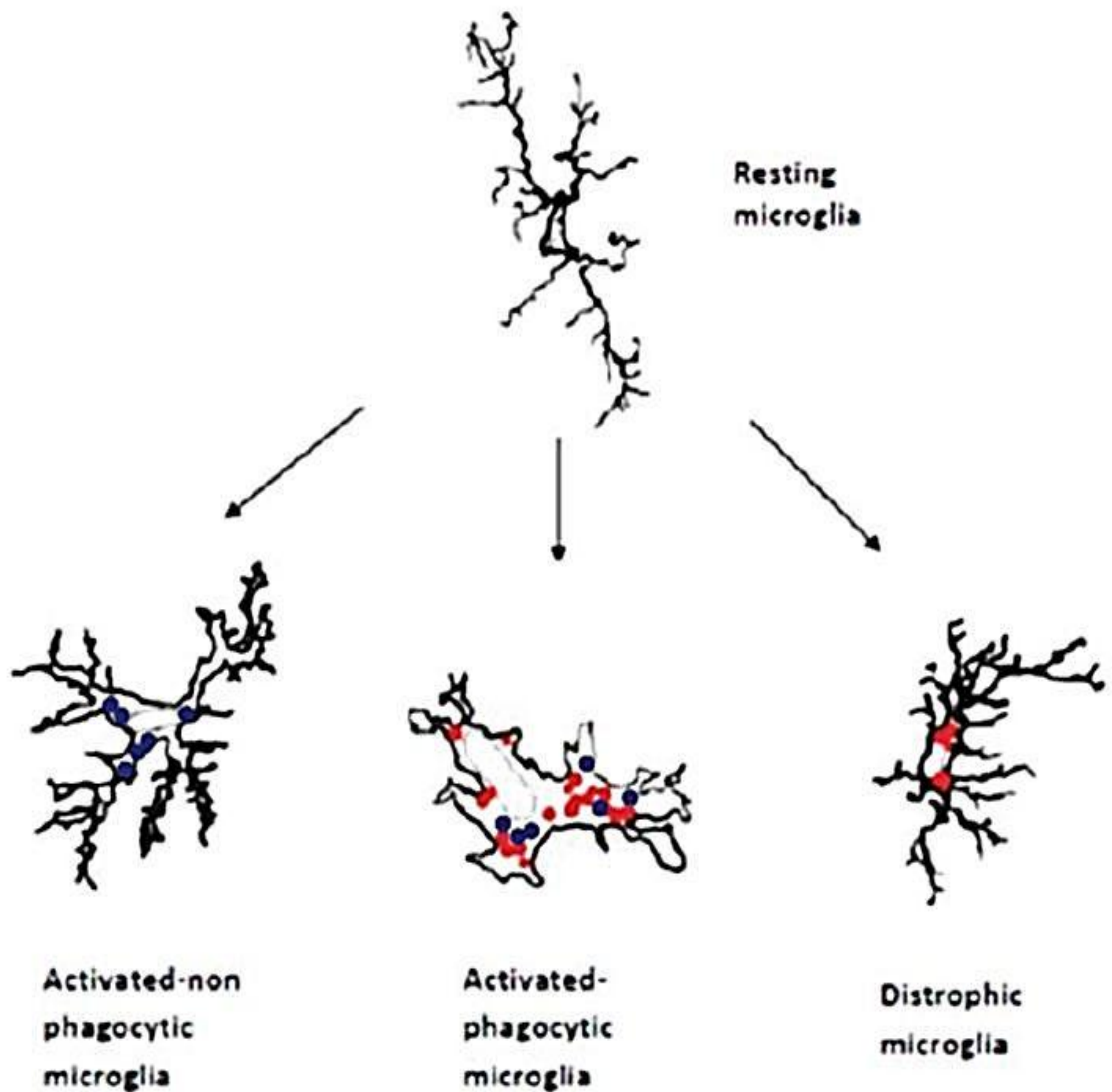

Figure 1-4. Microglia phenotypes. In normal conditions, microglia assume a resting phenotype, when stimulated activated-non phagocytic or activated-phagocytic phenotype can be expressed, both these phenotypes present hypertrophic cell body and in phagocytic phenotype a reduction of branching is present. 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 and CD11β marker expressed in active phenotypes. [Adapted from Streit and Xue 2009.Life and death of microglia. pag. 373 Fig.1]

1.2.2 Microglia as CNS Immune Cells

Streit (2002) elegantly summarizes how external stimuli could activate microglia resident to the brain. Because the skull, meninges and blood brain barrier 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 blood brain barrier separates the circulating immune system from the recently recognized neuroimmune system. In fact, until recent years, the CNS was thought 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 blood brain barrier (Banks *et al.* 1991, Banks *et al.* 2001) while others are actively transported by proteins through circumventricular organs. Cells of the blood brain barrier 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 blood brain barrier 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.

1.2.3 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.

1.3 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 of at least 32 cytokines in the hippocampus of adult mice, which 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

2.1 Aims and significance of experiments

Transient illnesses, such as common flu, impose a significant burden on society, and cause a significant economic impact, i.e. the loss of productivity caused by both absenteeism and by staff functioning at reduced capacity even after they have returned to work (Keech & Beardsworth 2008). A number of treatment options exist for transient illnesses and should be given serious consideration in an attempt to reduce the economic burden on society. Moreover the additional effect of cognitive impairment has not been widely studied. The origin of this impairment that follows transient inflammation is not completely known but previous publications have shown a reduced neurogenesis following transient inflammation. Furthermore reduction in neurogenesis has been correlated with impaired performances in hippocampal related tasks. Previous studies from Ormerod lab showed how 5 mg/kg of LPS injected intraperitoneally decrease neural progenitors differentiation and impairs hippocampal related spatial memory weeks after administration when newly generated neurons would have become part of the neural network. The effect of reduction in neurogenesis and the memory impairment have shown to be restored from different non-steroidal anti-inflammatory drug (NSAID), and in particular indomenthacin provides the best protection to both effects of LPS on neural progenitor cells (NPCs) and memory. These data suggest that a modulation of cytokines in the neurogenic niche could improve outcomes of neuroinflammatory processes. For this reason we concentrated our attention on NPCs fate and differentiation and on microglia. Microglial cells in particular are responsible for the production of several pro- inflammatory cytokine and they play a key role in the reduction of neurogenesis as shown from independent studies from Monje and Ekdhal's (Monje et al. 2003, Ekdahl et al. 2003) as well as Ormerod research group (Asokan & Ormerod 2009). Previous studies from Monje and colleagues have been done *in vivo* in order to show reduction of hippocampal neurogenesis due to LPS and *in vitro* where the effect of microglia on neurogenesis was derived from studies involving the identification of cytokines produced by microglia when stimulated by LSP. For these reason we decided to perform all our studies *in vivo* and access the effects of peripheral LPS induced inflammation on both hippocampal neurogenesis and microglia activation in the neurogenic niche of the hippocampus. Furthermore *in vitro* experiments have several

limitations. First the use of cell lines is still under discussion since the genetical modification of cells in order to immortalize them might engender secondary modifications to the cell behavior, second our preliminary studies *in vitro* on microglia were developed using as culture media DMEM (Dulbecco's Modified Eagle Medium) and 20% FBS (Fetal Bovine Serum). FBS in particular increases cellular proliferation but introduces high experimental variability due to uncertainty in his composition. Third microglia might be altered just by passaging and cell culture practices as we found in our preliminary experiments, were we analyzed the activation of microglia in vitro. In particular in those *in vitro* experiments we analyzed activation induced by addiction to the culture media of LPS for few hours followed by restoration of LPS-free media. To have significant results we had to wait 2 days after subculture in order to avoid effects due to cell passaging (by trypsinization). This preliminary study is still under completion in Ormerod lab but preliminary results revealed altered expression of activation immune-markers as CD11β and CD68 and this experiment will also reveal what cytokines are produced by microglia when stimulated by LPS *in vitro*.

Being aware of limitations of *in vitro* experiments we performed all experiments reported in this thesis *in vivo* on mice. In particular we injected animals with LPS (treatment group) and saline solution (control group) and subsequently injected animals with BrdU, a thymidine analog that gets incorporated in animals DNA for few hours after injection and can be revealed trough immunohistochemical procedures after brain tissue harvesting. BrdU labels cells during DNA synthesis of mitotic process allowing us to identify new cells. Then we evaluated effects of LPS on neurogenesis analyzing neural differentiation of new cells in the subgranular zone of the hippocampus (the hippocampal neurogenic niche). As described in previous chapter we used NeuN, a mature neural marker and DCX an early neural marker in order to identify new cells that had started differentiating into neurons. At the same time we evaluated microglia activation considering the expression of CD11β and CD68, respectively an activation marker and a phagocytic marker. We identified microglia with IBA-1 marker and measured cell body area an indicator of cellular morphological changes knowing that hypertrophy is associated with activated phenotype (Streit & Xue 2009).

2.2 Experiment preparation

Before starting our experiments we performed a systematic planning of timing, LPS dose and several test staining in order to quantify the optimal antibody dilution.

2.2.1 LPS dose

In order to identify the correct dose of LPS that needs to be injected in mice in this experiment preliminary studies have been performed by Ormerod and colleagues. At first a dose of 1 mg/kg was chosen following literature on LPS administration on rats. This dose revealed no reduction in neurogenesis and the inflammation induced was really low as showed from animal behavior. The dose had to be increased up to 5 mg/kg in order to have a moderate inflammatory process that lasts few days, as shown from dehydration and lack of appetite in mice. Furthermore 5 mg/kg dose reduces neurogenesis and engenders memory impairments (Ormerod et al. under review).

2.2.2 Timing

The decision of timing of the experiments and in particular the time gap between LPS administration, BrdU injection and killing of animals has been planned before starting experiments. Preliminary studies performed in Ormerod lab revealed a higher cytokine production in the hippocampus during the first 4 days after LPS intraperitoneal injection. These data were obtained harvesting blood serum and hippocampus of saline perfused animals. Cytokines production appeared to be different in serum compared to hippocampus, index of the activity of neural parenchyma and cytokine production in the CNS. Consequently the CNS parenchyma plays an active role in the production of neuroinflammatory molecules and the process is not limited to a direct afflux of proinflammatory cytokines trough the blood brain barrier. The activity and altered cytokine production showed to be present from few hours after LPS administration and slowly reduce till disappearing within 4 days (Asokan & Ormerod 2009). For these reasons a first experiment was performed to evaluate short term effects of intraperitoneal LPS on microglia phenotype, cells that have shown to have a high cytokine production capability. Animals were injected with LPS/saline and then killed at 5, 24, 48, and 96h, in order to have a complete overview of the effects during the first four days after induced inflammation starts. As described before microglia have the capability of producing pro-

inflammatory cytokines and there is a strong presence of microglia in the hippocampus, this is the reason of the importance evaluating their phenotype within the first 4 days (see figure 2-1).

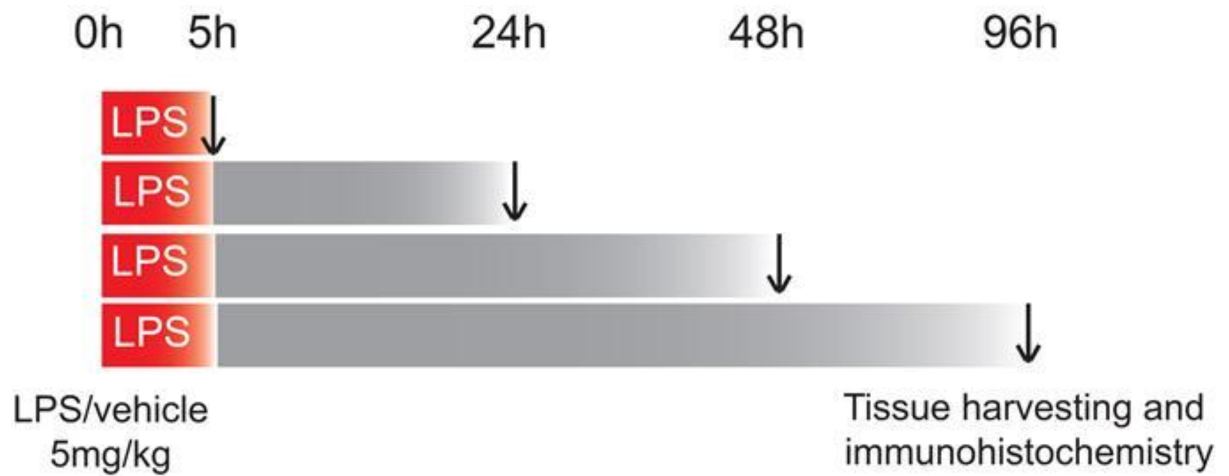

Figure 2-1.Timing of short term experiment on microglial cells. Time zero corresponds to LPS or saline (control) injection, arrows indicate to time of death.

A second experiment was performed in order to evaluate long term effects on microglia and neurogenesis of LPS induced neuroinflammation. Preliminary studies effectuated by Ormerod research group revealed the possibility of a chronic activation of microglia at 1 month after LPS injection, since a possible modification of the morphology of microglia had been evaluated. This is why we analyzed microglia phenotype at 1 and 4 weeks after LPS administration (see Figure 2-2).

Figure 2-2.Timing of long term experiment on microglial cells. Time zero corresponds to LPS or saline (control) injection, arrows indicate to time of death.

Finally in the second experiment we also evaluated the long term effects of LPS on neural differentiation. The timing of this experiment is more delicate since results depend on when and how many times BrdU is injected and on the time of death of the animal. As explained we decided to use a dose of 5 mg/kg of LPS injected intraperitoneally in order to engender a peripheral inflammatory process (time zero). BrdU (thymidine analog) can be injected once or multiple times. In literature several studies used multiple injections in order to label a higher number of dividing cells, on the other had the use of multiple injections reduces the time resolution the outcome of the experiment. This because with a single injection we label cells dividing up to few hours after BrdU injection and subsequent injections introduce new time windows in which dividing cells incorporate BrdU in their DNA, thus, with multiple injections, after immunohistochemical procedures we wouldn't be able to distinguish between cells that had incorporated BrdU in different time windows. In our experiment we wanted to evaluate effects of LPS on neurogenesis weeks after LPS injection. We decided for 3 BrdU injection for animals killed at 2, 3 and 4 weeks after LPS. BrdU injections were started always one week before killing the animal. The time lapse between BrdU injection and killing of the animal is extremely important because BrdU labels and gets incorporated in new cells that keep on growing and differentiating till the death of the animal. We set the time lapse between the first BrdU injection and death at one week in order to provide cells sufficient time to proliferate and differentiate and consequently we used mature neural markers NeuN and early neural marker DCX that are expressed at one week after neural progenitor cells generation.

A further control to our experimental setting was provided by a group of animals (both LPS and saline treated) on which we evaluated short term effects of LPS on neurogenesis. This group received a single injection of BrdU in order to have a higher time resolution and to have results directly comparable with our preliminary studies and other literature sources. In particular from previous studies we already dispose of data on short term effects of LPS on neural differentiation derived from different independent experiments revealing reduction of neurogenesis in the short term due to LPS.

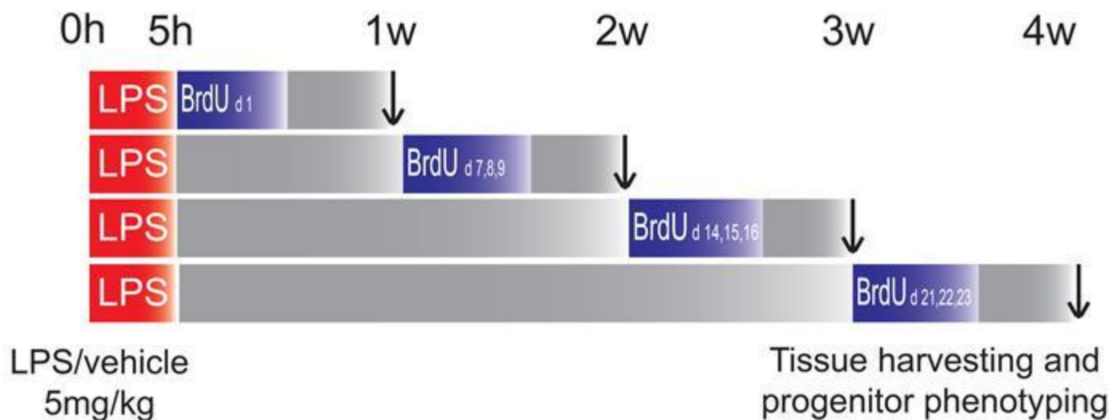

Figure 2-3. Timing of long term experiment on NPCs differentiation. LPS or saline (control) was injected at time zero, blue indicates BrdU injection and arrows indicates time of death of mice.

2.2.3 Immunohistochemistry

Immunohistochemistry consist in the detection of proteins and their localization on fixed tissue. A tissue blocking solution is used at the beginning of the process in order to reduce background staining. Proteins are then detected by a primary antibody that establishes a connection with the target protein. Then after washing out the excess of primary antibody a secondary antibody conjugated with a fluorophore is used (see Figure 2-4). After this treatment a particular protein can be identified through fluorescence microscopy.

The immunohistochemical process has several critical steps that might negatively affect the outcome of the procedure. The major problem is background staining that might be due to a weak targeting, excessive concentration of the antibody in the staining solution or to the absence of an effective blocking of the tissue performed before the staining process. Auto-fluorescence can also negatively affect the outcome of the immunohistochemical process. In particular paraformaldehyde needs to be wormed up during its preparation to facilitate the dissolution of its components but an excessive temperature might be cause of auto-fluorescence of paraformaldehyde compromising all immunohistochemical procedures. To prevent auto-fluorescence paraformaldehyde was freshly made before each perfusion and stored at 4 C° only for few hours before use. Furthermore an excessive presence of blood in vasa might be cause of fluorescence and might be recognized as a target from several primary antibodies causing artifacts in the fluorescence image. All these problems were avoided trough an accurate preliminary study

and to several test staining that we performed before final experiments reported in this thesis. In particular, we evaluated the optimal concentration of all antibodies used in order to have a clear identification of targets and reduce to the minimum background fluorescence.

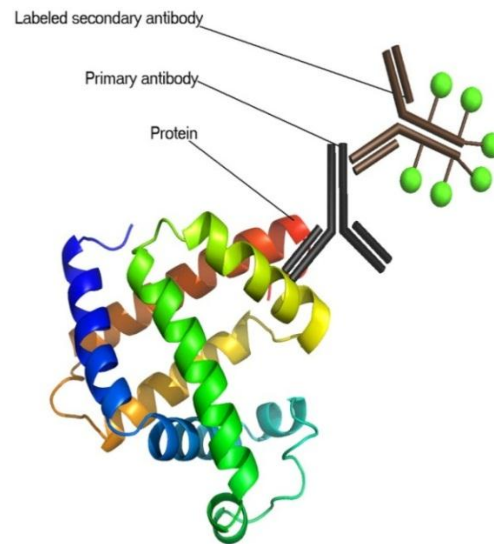

Figure 2-4. Protein linked to a primary antibody that is then linked to a secondary fluorescent specific antibody in order to allow the detection of the protein with fluorescence microscopy [Adapted from: www.histalim.com].

In order to evaluate what antibody would have given a better result we analyzed literature on microglia. It has to be noticed that antibody have animal specificity and different results can derive from the use of antibodies on mice instead of rats (as used by Streit et al 2009 and described in the first chapter). For these reasons we performed several test staining using different antibodies and different concentration on mice tissue. The antibodies and concentrations used are reported in the table 2-1.

Primary antibodies used in immunohistochemical studies on microglia			
Primary antibody	Specific use	Dilution	Supplier
IBA-1	Microglia cells identification	1:1000	Wako, Richmond, VA
CD68	Microglia cells phagocytic activity	1:500	ABD Serotec, Raleigh, NC
CD11β	Microglia cells activation	1:100	BD Bioscience, San Jose, CA
DAPI	Cell nuclei identification	1:3000	EDM Millipore, Billerica, MA

Table 2-1. Primary antibodies used to identify microglia and access their activation status and phagocytic activity.

The use of specific antibodies in order to evaluate the fate and differentiation of neural progenitors is based on previous literature, in particular in Ormerod lab extensive study of neural progenitors have been developed and at one week of maturation of new cells (time lapse between first BrdU injection and death of the animal) the use of NeuN and DCX has shown interesting results and allows to distinguish NPCs that have started the process of differentiation into neurons. The list of antibodies used to evaluate neural progenitors phenotype is shown in table 2-2.

Table 2-2. Primary antibodies used in immunohistochemical studies on neurogenesis			
Primary antibody	Specific use	Dilution	Supplier
Anti-BrdU	Identification of new cells	1:500	ABD Serotec, Raleigh, NC
NeuN	Mature neural marker	1:500	Millipore, Billerica, MA
DCX	Early neural Marker	1:500	Santa Cruz Biotech, Accurate, NJ
DAPI	Cell nuclei identification	1:3000	EDM Millipore, Billerica, MA

Table 2-2. Primary antibodies used to identify new cells and to access their phenotype.

2.3 Instrumentation

2.3.1 Perfusion pump

A peristaltic pump was used in order to perform the sacrifice perfusion. Perfusion was first described by Palay et al. (Palay et al. 1962), they used gravity pressure in order to drive a first prewash with physiological saline followed by fixative. This method first used by Palay does not always provide a complete washing of white blood cells from circulation. For these reason we opted for the use of a peristaltic pump that provides a constant flow rate with no control on pressure. Pump settings were determined from preliminary results effectuated by Professor Ormerod in order to have a complete and uniform washout of the brain tissue (see Figure 2-5). The flow rate had to be determined experimentally since the cardiovascular resistance is highly variable between species and it presents high variations due to gender, exercise state and previous history of the animal, hence with our experimental set up it was not possible to calculate with precision what flow rate should be maintained in order to maintain a correct pressure in the capillary. Pressure inevitably varies during constant flow perfusion but the experimental value used in our experiment based on preliminary studies prevented capillary damage and provided a complete washout of erythrocytes that might otherwise cause artifacts in the fluorescence imaging procedures.

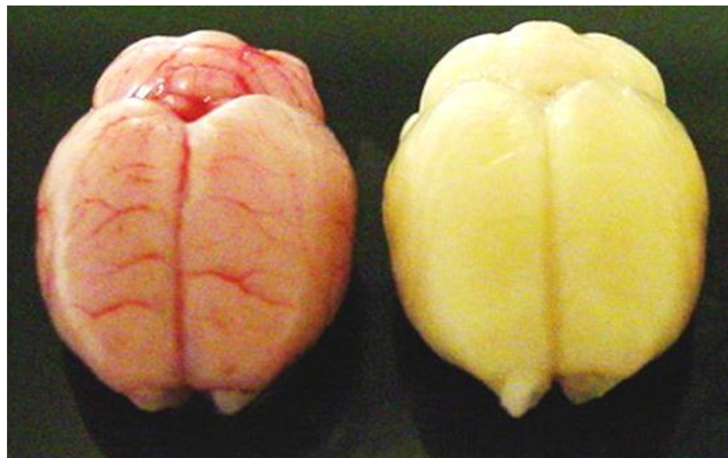

Figure 2-5 Mouse brains showing a correct perfusion (on the right) completely washing out erythrocytes and a non-perfused brain (on the left) [Adapted from www.leica-microsystems.com].

2.3.2 Microtome

A microtome (Figure 2-6) is an instrument used to cut extremely thin slices of tissue. In particular 40 um sections of brain tissue were cut in our experiments. The microtome is composed by a moving stage on which brain tissue is cryo-fixed. A wedge shaped extremely sharp blade cuts small slices of tissue that are removed one by one and placed in a cryopreservative solution (30% ethylene glycol, 30% glycerol and 30% phosphate buffer) and stored for subsequent immunohistochemical processing.

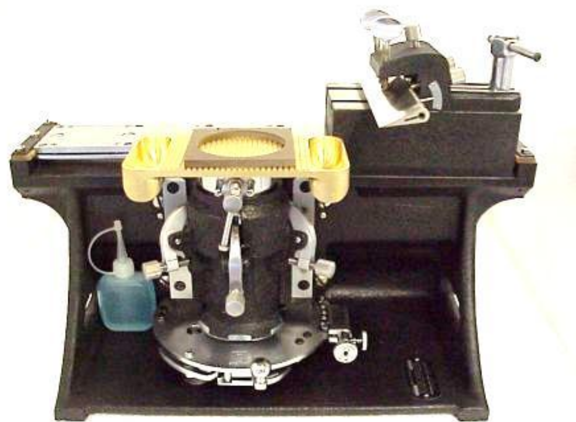

Figure 2-6. Microtome used from brain slicing (American Optical Corporation, Buffalo, NY).

2.3.3 Confocal microscopy

Confocal laser scanning microscopy has an important feature that distinguishes it from the traditional conventional fluorescence microscope: the confocal aperture or pinhole. Ideally the pinhole has infinitely small aperture thus the detector is focused on a particular point trough the 3D coordinates of the object observed. The pinhole is located between the image plan and the object and as a consequence the detector can only detect light that has passed the pinhole. The pinhole is located on a plane conjugate to the focal plane of the objective. Subsequently the light coming from planes above and below the focal plane of the objective is stopped by the pinhole therefore just a particular point on the Z-plane can be identified (see Figure 2-7). This characteristic is not present in a common fluorescence microscopy where all the Z-planes are condensed in one. For our experiments the use of confocal microscopy is extremely important since it allows a more accurate identification and counting of neural progenitors and microglia. In particular, a single

tissue slice might contain more than one cell through its Z-planes and thanks to confocal microscopy we are able to distinguish different cells that would have been superposed using traditional microscopy techniques. Furthermore different proteins identified in our experiments have a different location on cells (cytoplasmic or on the cell membrane) and the localization of flourophores on the cell can be used in order to have a further confirmation of a correct staining.

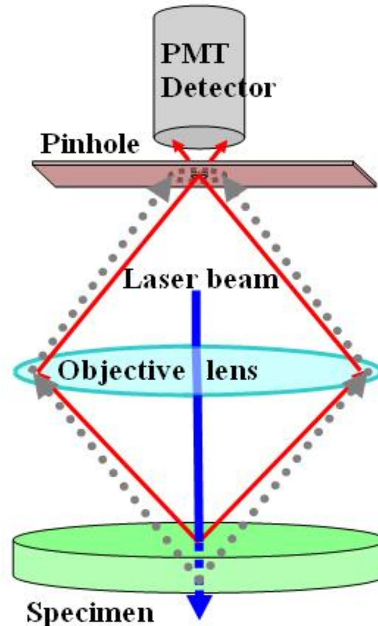

Figure 2-7. Simplified representation of the effect of a confocal microscope. In particular is shown how points on the focal plane can reach the detector meanwhile light coming from planes above or below the focal plane is arrested from the pinhole and is not detected. [modified from: http://www.huji.ac.il].

We used a Zeiss LSM 710 confocal microscope that has excitation laser lines at 405, 458, 488, 514, 561 and 633nm. It can provide three separate spectral detection channels and is capable of detecting many standard fluorophores, including DAPI, FITC, CY3 and CY5 as we used in our experiments. Thanks to the confocal properties we can acquire several Z-planes images and then through image processing software we can superpose them, or create a 3D reconstruction of the tissue. In our experiments we used single planes. In order to identify a single cell we moved in all planes where the cells is present in order to have a precise evaluation of markers expressed by a particular cell, in our case neural progenitor cells or microglia as explained in details in next sections.

A further tool of image processing we used is the colocalization function. The colocalization function permits the interactive analysis of two different channels corresponding to two different fluorophores secondary antibodies conjugated to primary antibodies IBA-1 and CD68, used respectively to identify microglia and to evaluate their phagocytic activity. Colocalization is defined by the presence of two different molecules at the same location in a specimen and in digital imaging it refers to colors emitted by fluorescent molecules detected by the same pixel image. The fluorophores used for the colocalization have been chosen in order to have an emission spectra well distinguished from each other and guarantee an accurate colocalization minimizing artifacts due to spectral overlap. Colocalization can be represented as scatter diagram with lines indicating thresholds as shown in Figure 2-8. On the scatter plot three regions can be distinguished as indicated in Figure 2-8, region 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 represents the area above threshold for IBA-1but below for CD68 indicating the presence of IB-1 staining not co-localized with CD68. Region 3 of the scatter plot represents the area of co-localized pixels and the last remaining region in the low left corner of the scatter plot(see Figure 3-8) contains pixels below threshold for both CD68 and IBA-1 The two lines represented in the plot are the thresholds of each channel. In the diagram, pixels having the same position in both images are considered pairs. Of every pair of pixels (P1, P2) from the two source images, the intensity of pixel P1 (corresponding to CD68) is interpreted as horizontal coordinate, and the intensity of pixel P2 (corresponding to IBA_1) as vertical coordinate of the scatter diagram. This way, the value of each pixel of the scatter diagram shows how often a particular pair of pixels has occurred. Ideally two identical images would therefore produce a diagonal line because only pixel pairs with the same intensity would occur. This diagram has been used at the beginning of the colocalization process in order to establish the optimal combination of thresholds. While choosing thresholds we want to have a sufficient number of colocalized pixels and at the same time colocalized pixels must have a correspondence with real colocalization that can be manually evaluated from the expert user, in order to verify correct parameters. In this thesis we analyzed the number of colocalized pixels present in region 3 of the scatter plot as an indicator of co labeling of microglia cells, identified with IBA-1 and labeled with phagocityc marker CD68.

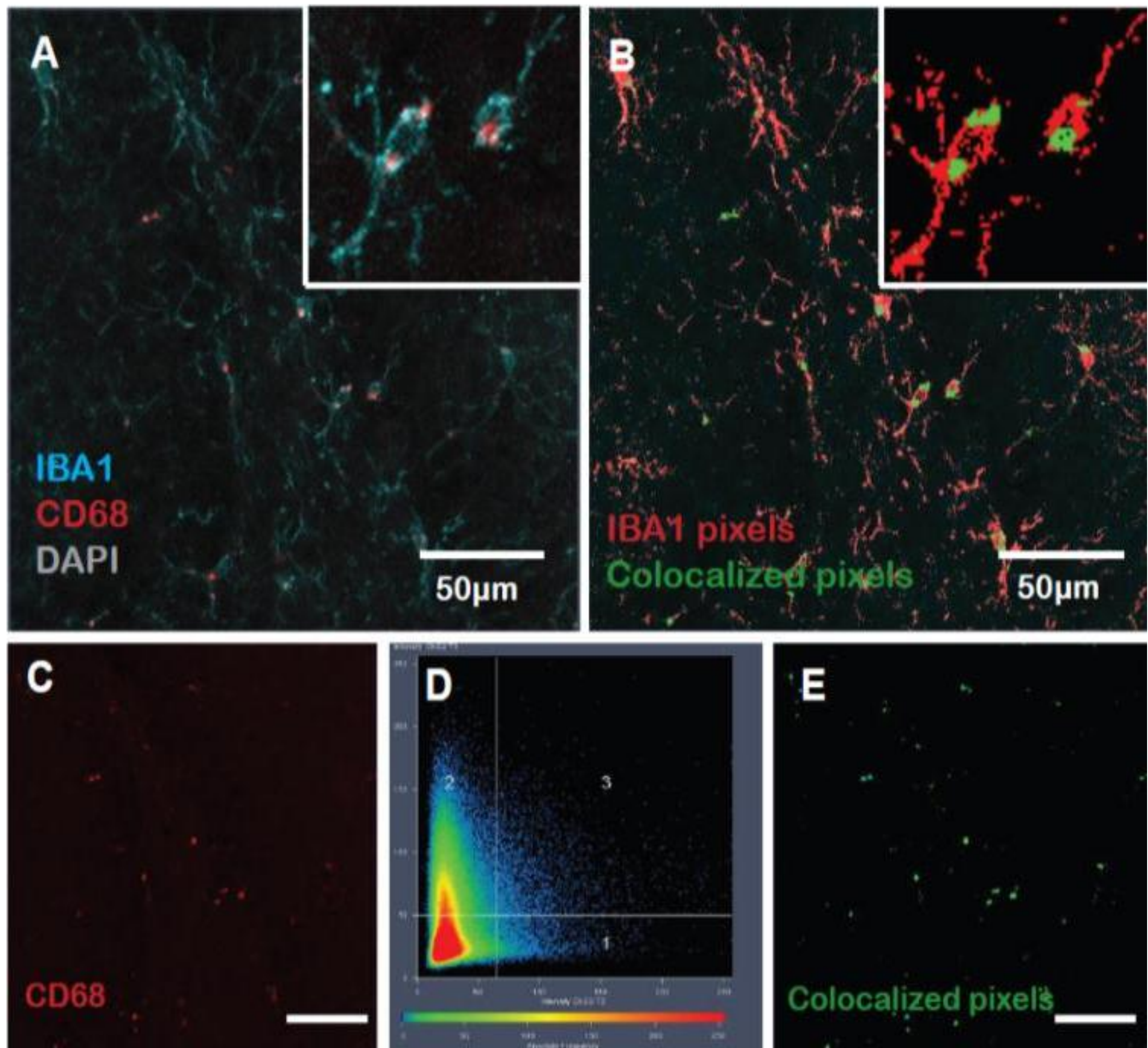

Figure 2-8. Co-localization parameters were chosen in order to minimize false co-localization of IBA-1/CD68 markers, where markers colors are arbitrarily set. (A) Actual picture of microglia cells (light blue: IBA-1) co expressing CD68 (red: CD68). (B) Identification of IBA-1 pixels over threshold intensity (red) and CD68 (green) co-localized pixels. (D) Scatter plot of pixel intensity, on the axis are represented IBA-1 and CD68 channels; region 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. Last region in the low left corner of the scatter plot contains pixels under threshold. (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).

2.4: Design of experiment

All tests and preliminary works were performed in order to find the best experimental set up for our experiments. In particular, we decided to divide the experiments in two parts. The first aimed to access short term effects of neural induced inflammation on microglia and a second to access long term effects of LPS induced inflammation on microglia and neurogenesis as shown in details in Figure 2-9 where is represented the division in two experiments the outcomes of each experiment. The area of interest is the hippocampus and in particular the subgranular zone where neurogenesis occurs in the adult. Experiments were performed in strictly controlled and repeatable experimental conditions.

We performed a first experiment focused on the first 4 days after LPS administration and we analyzed microglia phenotype considering expression of particular proteins index of activity and the evaluation of cell body area. We evaluated the proportion of cells expressing CD11β and CD68 respectively a marker present in activated microglia and a phagocytic marker. Due to the presence of the totality of microglia cells (identified with IBA-1 microglial marker) co-expressing CD68 we decided to continue the investigation with a pixel intensity analysis of colocalized IBA-1/CD68 pixels, as described previously, in order to be able to distinguish possible low differences in expression of the phagocytic marker due to LPS treatment. Furthermore we measured cell body area to quantify possible morphological variations index of an active phenotype.

A second experiment was performed to access effects in the long term after LPS administration on microglia and neurogenesis. We analyzed microglia phenotype as described before and we labeled dividing cells with BrdU, a thymidine analog that gets incorporated into DNA of dividing cells. New cells phenotype was evaluated analyzing the expression of DCX and NeuN, respectively an early progenitor marker and a mature neural marker. As control of the experimental set up we evaluated neurogenesis at 1 week in order to be able to directly compare our results with literature studies reporting effects of LPS on neurogenesis at 1 week. Protocols were strictly followed and in next section a detailed description is provided.

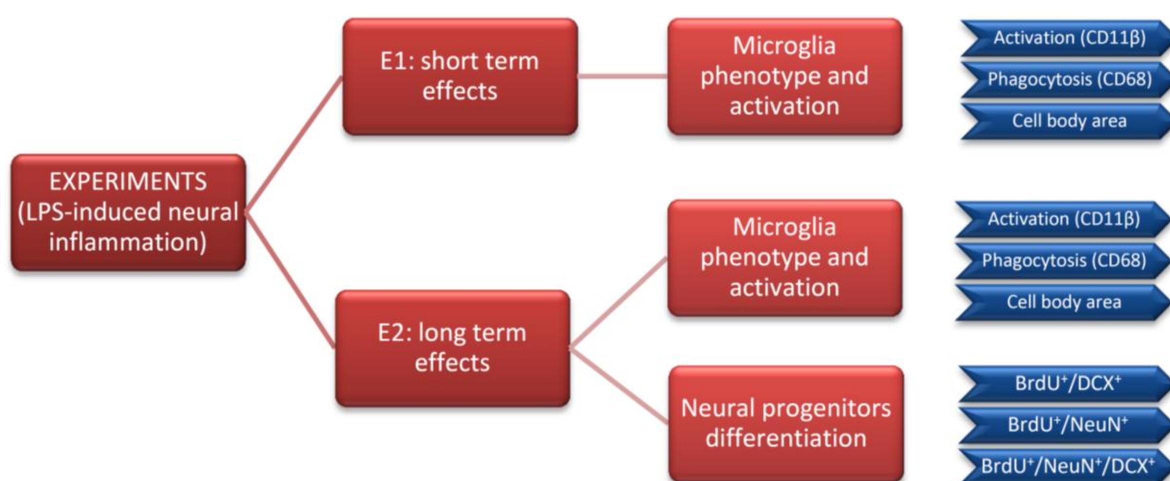

Figure 2-9. Diagram representing a scheme of the two experiments developed in vivo. In the first experiment (E1) we accessed the effects of LPS injected intraperitoneally on microglia activation and phenotype within the first 4 days. In particular we evaluated microglia activation and phagocytosis considering the expression of respectively CD11β and CD68. In the second experiment (E2) we evaluated microglial and neural phenotype in a period from 1 to 4 weeks after LPS administration. To access neural phenotype we measured the proportion of BrdU+, BrdU+/DCX+, BrdU+/DCX+/NeuN+, BrdU+/NeuN+ cells.

2.4.1 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, USA) 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.

2.4.2 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).

2.4.3 LPS 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 of LPS was injected intraperitoneally on Day 0. All mice were injected with saline solution subcutaneously at 24h after LPS to prevent dehydration.

2.4.4 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.

2.4.5 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.

2.4.6 Experiment 1: Short Term Effects of LPS on Microglia

Experiment 1 evaluated the short-term effects of LPS injection on microglial morphology and activation state. A total of 72 animals were used paying the maximum

attention to the reduction to the minimum necessary of mice. 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.

2.4.7 Experiment 2: Long Term Effects of LPS on Microglia and Neurogenesis

Experiment 2 measured the effects of LPS administration on neurogenesis and on microglial activation weeks after infection. We used a total of 40 animals for this second experiment and in particular in order to reduce the number of animals, tissue from mice killed at 1 week an 4 weeks have been used for both neural progenitor and microglia phenotype evaluation. 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 $IBA\text{-}1^+$ cells that co-expressed CD11β and/or CD68 and by examining their cell body areas.

2.4.8 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.

2.4.9 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 for 10 min to reveal cellular nucleus (1:3000; Calbiochem, EDM Millipore, Billerica, Massachusetts) before being cover-slipped under PVA-DABCO (a glycerol based cover-slipping solution).

2.5: Analysis

2.5.1 Data analysis

As outcome of our experiments we recollected data about microglia activation, phagocytic activity and morphology in the short term and microglia and neural progenitor cells phenotype in the long term. At the end of our experiments we dispose of data concerning the proportion of IBA-1 microglia expressing CD11β (activation marker), and the pixel intensity CD68 (phagocytic activity marker) pixels colocalized with IBA-1 (microglial marker) both in the short and long therm. We recollected data concerning new cells (expressing BrdU, thymidine analog) phenotype in the long term and in particular the proportion of cells expressing $BrdU^+$, $BrdU^+/DCX^+$, $BrdU^+/DCX^+/NeuN^+$ and $BrdU^+/NeuN^+$ in order to have an indicator of the differentiation rate of progenitor cells.

In next section we describe how data have been recollected and the statistical analysis we applied.

2.5.2 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 (subgranular zone), 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 $IBA\text{-}1^+$ cell and then the proportion of activated microglia ($IBA\text{-}1^+$ versus $IBA\text{-}1/CD11\beta^+$ cells) or phagocytic (IBA-1 versus $IBA\text{-}1/CD68^+$ cells) was assessed. Because all $IBA\text{-}1^+$ 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. 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 $IBA\text{-}1^+$ cells per animal was selected and the area of the cell

determined using the Zen Zeiss Confocal Microscope processing software perimeter tool (see Figure 2-10).

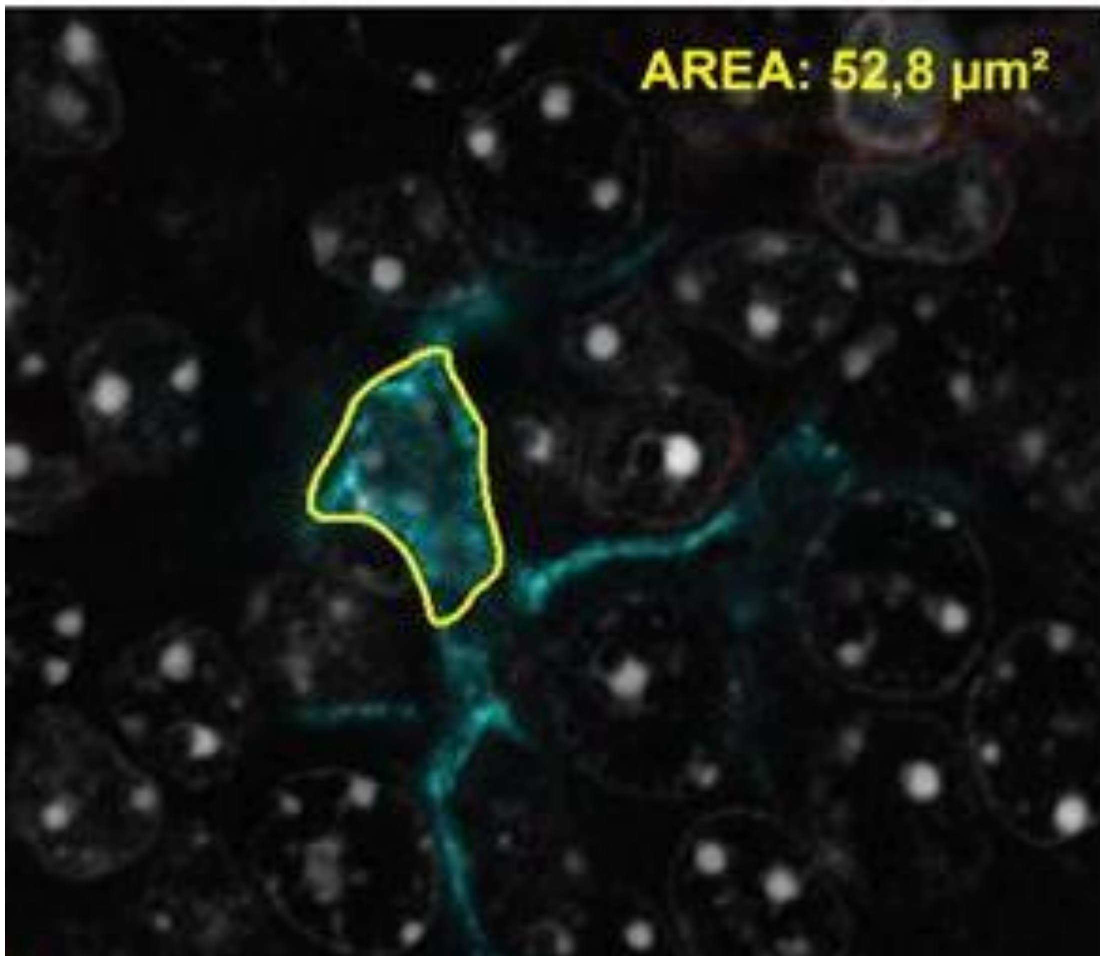

Figure 2-10. Cell body area selection and measurement using Zeiss software. In particular this image was take with Zeiss confocal microscope and shows a microglial cell, identified with IBA-1 (light blue: IBA-1) and confirmed from the presence of nucleus (gray: DAPI).

2.5.3 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.

2.5.4 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). We performed an ANOVA test since we had to compare the mean of several distinct groups related to each other. In particular we tested the null hypothesis that all groups are random samples of the same population. This null hypothesis implies there is no effect of any of the analyzed variables and the rejection of this hypothesis implies there actually is an effect of treatment and/or time on microglia and new cells phenotype. The use of this null hypothesis is done in order to limit the rate of type I errors (false positives). A difference was considered statistically significant when p-value was less than 0.05 and in this case the null hypothesis was rejected. Newman Keul's post hoc test was used to revealed group differences when appropriate. Post hoc examination was performed when ANOVA revealed statistically significant differences and the use of a post hoc test provides an effective control of type 1 error rate. Statistical analyses were performed using STATISTICA software (StatSoft; Tulsa, OK). Values are expressed in graphs as mean ± SEM.

CHAPTER 3
RESULTS

In the presented experiments we evaluated the effects of a peripheral LPS induced neuroinflammation in the short term on microglia. We evaluated the presence of phagocytic activity, the proportion of active microglia expressing CD11β and the morphology of microglia. Then we analyzed long term effects of neuroinflammation on microglia and neural progenitors phenotype in particular to evaluate new cells phenotype we considered the proportion of cells expressing $BrdU^{+}$, $BrdU^{+}/DCX^{+}$, $BrdU^{+}/DCX^{+}/NeuN^{+}$, $BrdU^{+}/NeuN^{+}$. In the next section results are presented in details.

3.1 Experiment 1: Short Term Effects of LPS on Microglia

3.1.1 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. We performed experiments on 3 animals per group and in the following tables pixel intensities are reported as percentage of control in order to directly compare different images (see Table 3-1). It has to be underlined that during the image processing all thresholds and lasers gains and intensities have been kept constant so that different images can be compared, as explained before in the methods section.

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 Table 3-2). These data suggest that peripherally administered LPS did not stimulate phagocytosis in microglia within the first 4 days after LPS administration as shown in Figure 3-2.

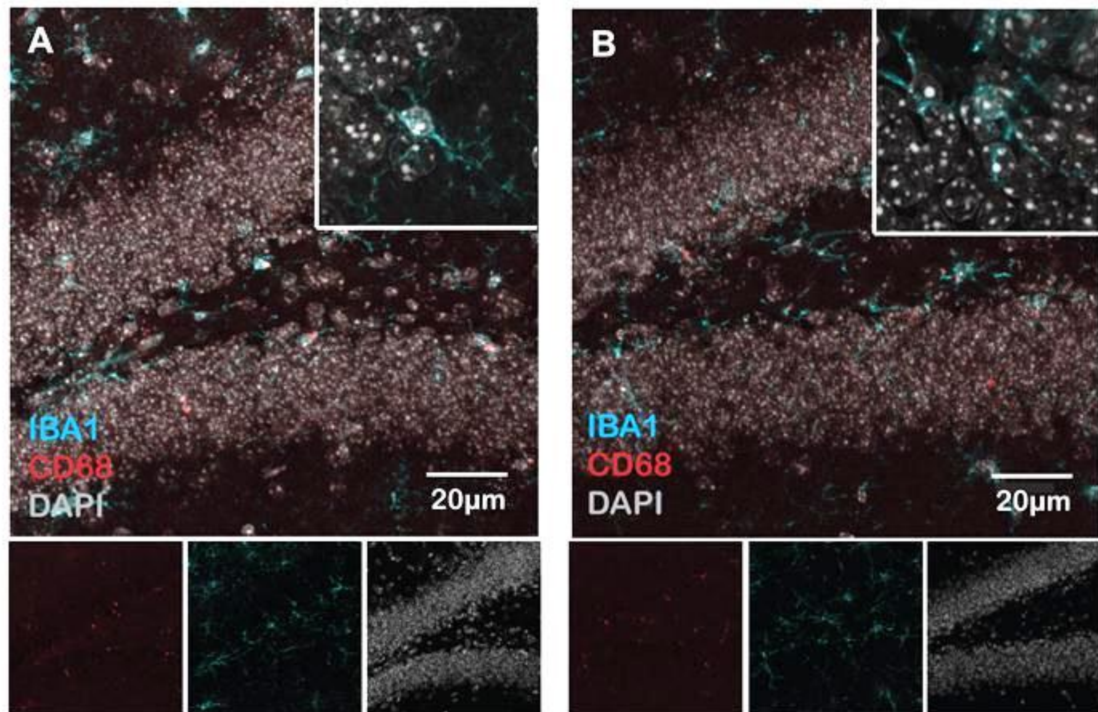

Figure 3-1. Low expression of CD68 (red) in both (A) control and (B) LPS animals killed 24h after LPS. IBA-1 (blue) and DAPI (gray) markers respectively for microglia and cellular nuclei.

Intensity of CD68 pixels colocalized with IBA-1			
	Mouse 1	**Mouse 2**	**Mouse 3**
Control 5h	102.7397	97.2602	100
LPS 5h	101.3699	104.4521	107.5342
Control 24h	107.4632	83.6664	108.8704
LPS 24h	82.2592	124.6228	82.2592
Control 48h	109.5951	98.5493	91.8555
LPS 48h	97.3666	90.6729	94.2208
Control 96h	105.8182	96.1983	97.9834
LPS 96h	105.8182	95.4822	90.1378

Table 3-1. Normalized pixel intensity values of CD68 (phagocytic activity marker) pixels colocalized with IBA-1 (microglial marker) at 5h, 24h, 48h and 96h.

Statistical analysis (ANOVA) of intensity of CD68 colocalized pixels		
Effect	**F**	**p-level**
Treatment	0.189439	0.669198
Time	0.241896	0.86583
Treatment and time	0.241896	0.86583

Table 3-2. No statistically significant effect due to treatment, time or interaction effect is revealed analyzing pixel intensity of CD68 pixels colocalized with IBA-1, index of absence of phagocytic activity in the short term.

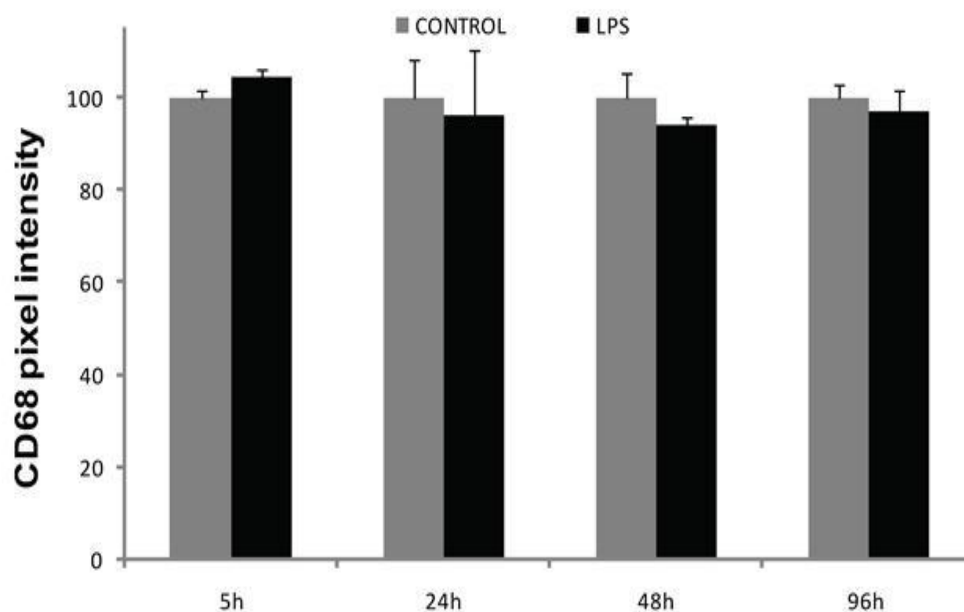

Figure 3-2. Pixel intensity of CD68 pixels co-localized with IBA-1 reveals absence of phagocytic activity (horizontal axis: time; vertical axis; percentage of control pixel intensity).

3.1.2 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 (see Figure 3-3).

Cell body areas are reported in Table 3-3 and on these data we performed an ANOVA analysis 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, see table 3-4). Specifically, larger microglia were found in the dentate gyri of LPS-treated mice (52.25±1.93) versus saline-treated mice (42.41±0.82, see Table 3-5). Independent of group (LPS or control) soma area increased with time after treatment such that they were larger at the 24 and 48h time points versus the 5h time point (p<0.01, see Table 3-6). 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 Table 3-7.

In conclusion microglia showed statistically significant hypertrophy of the cell body at 24h and 48h after LPS intraperitoneal injection compared to control group injected with saline solution as shown in Figure 3-4.

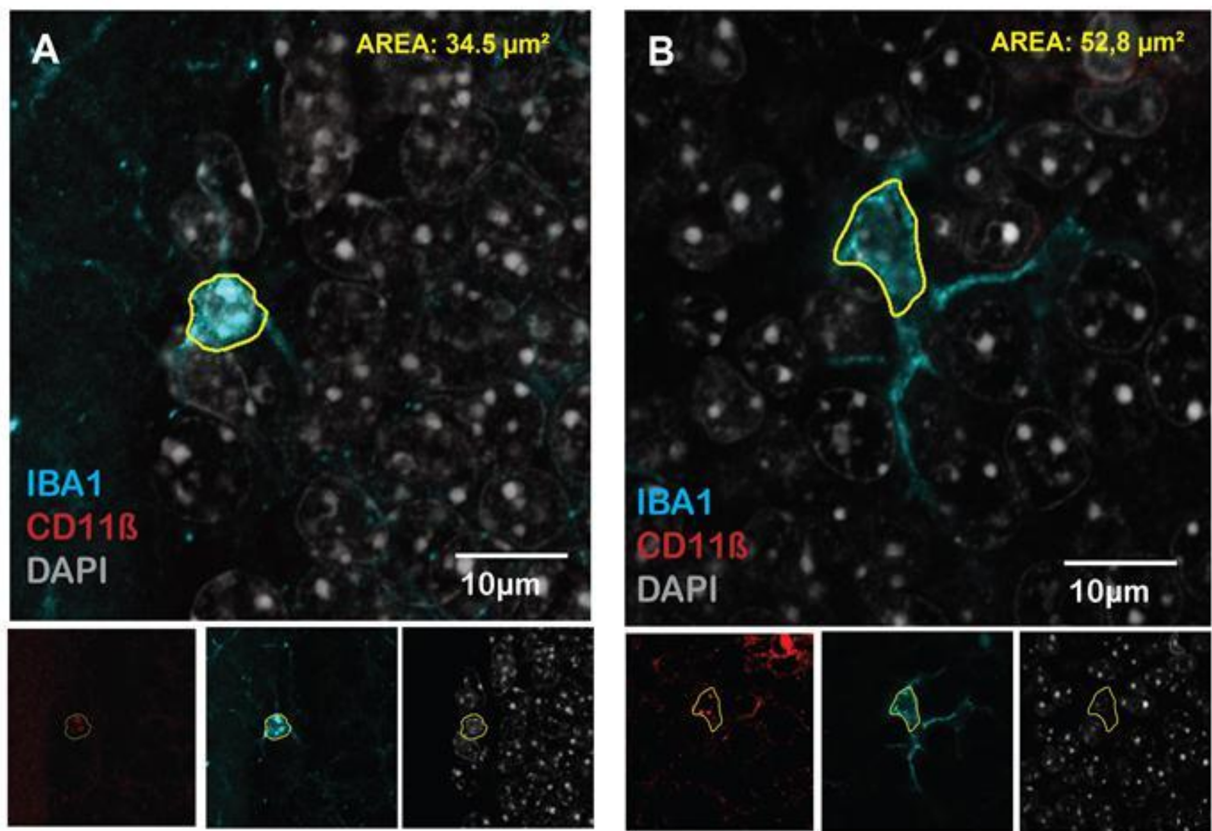

Figure 3-3. Cell body area showing hypertrophy due to LPS administration. (A) Mouse killed 24h after saline solution administration (control), showing a cell body area of 34.5µm^2 (perimeter selected in yellow) and (B) mouse killed 24h after LPS injection showing an area of 52.8 µm^2. Microglial cells identified with IBA-1 (light blue: IBA-1) and the presence of nucleus (gray: DAPI), CD11β activation marker red (red: CD11β)

Microglia cell body area				
	Mean (n=3)		Standard error	
	CONTROL	LPS	CONTROL	LPS
5h	40.99	42.11	0.45	1.46
24h	41.81	58.96	1.45	4.00
48h	45.90	59.69	3.75	2.69
96h	40.80	48.24	1.00	2.75

Table 3-3. Microglia cell body area expressed in mm^2. An hypertrophy of the cell body at 24h and 48h due to LPS administration has been confirmed from statistical analysis.

Statistical analysis (ANOVA) of microglia cell body area		
Effect	**F**	**p-level**
Treatment	31.01778	**0.000042**
Time	8.527869	**0.0013**
Treatment and time	3.996359	**0.026659**

Table 3-4. Short term effects on microglia cell body area reveal an effect of time, treatment and an interaction effect, as revealed from an ANOVA.

Post hoc analysis, main effect: Treatment		
	Control	LPS
Mean	42.37417	52.25000
Control		**0.000192**
LPS	**0.000192**	

Table 3-5. Newman Keul's post hoc test revealed effect of treatment on microglia morphology in the short term.

Post hoc analysis, main effect: Time				
	5h	**24h**	**48h**	**96h**
Mean	41.5533	50.3833	52.7933	44.5183
5h		**0.0076**	**0.0020**	0.2544
24h	**0.0076**		0.3509	**0.0327**
48h	**0.0020**	0.3509		**0.0119**
96h	0.2544	**0.0327**	**0.01199**	

Table 3-6. Newman Keul's post hoc test revealed effect of time on microglia morphology in the short term; in particular a statistically significant difference is present between 24h-48h, 24h-96h and 48h-96h groups. The effect of time might be partially due to the fact that in this post hoc test we are considering Control and LPS groups together, for this reason it is interesting evaluating the interaction effect (see Table 3-7).

Post hoc analysis, main effect: Interaction Time x Treatment								
	5h	24h	48h	96h	5h	24h	48h	96h
	Control	Control	Control	Control	LPS	LPS	LPS	LPS
Mean	40.99	41.81	45.89	40.79	42.11	58.95	59.69	48.24
5h Control		0.8209	0.5274	0.9566	0.9468	**0.0014**	**0.0013**	0.2908
24h Control	0.8209		0.4974	0.9562	0.9330	**0.0016**	**0.0015**	0.3035
48h Control	0.5274	0.4974		0.6138	0.3020	**0.0055**	**0.0065**	0.5183
96h Control	0.9566	0.9562	0.6138		0.9820	**0.0017**	**0.0015**	0.3356
5h LPS	0.9468	0.9330	0.3020	0.9820		**0.0013**	**0.0013**	0.2258
24h LPS	**0.0014**	**0.0016**	**0.0055**	**0.0017**	**0.0013**		0.8389	**0.0083**
48h LPS	**0.0013**	**0.0015**	**0.0065**	**0.0015**	**0.0013**	0.8389		**0.0139**
96h LPS	0.2908	0.3035	0.5183	0.3356	0.2258	**0.0083**	**0.0139**	

Table 3-7. Newman Keul's post hoc test revealed an interaction effect of Time and Treatment. In particular 24h and 48h groups show a statistically significant hypertrophy of the cell body due to LPS treatment compared to control. As expected all control groups do not show statistically significant differences between each other (dark gray square $p>0.05$), meanwhile 5h LPS and 96h LPS groups appear both to be different from 24h LPS and 48h LPS groups (light gray box) because we see an effect of LPS administration only at 24h and 48h meanwhile no effect appears to be present at 5h and 96h.

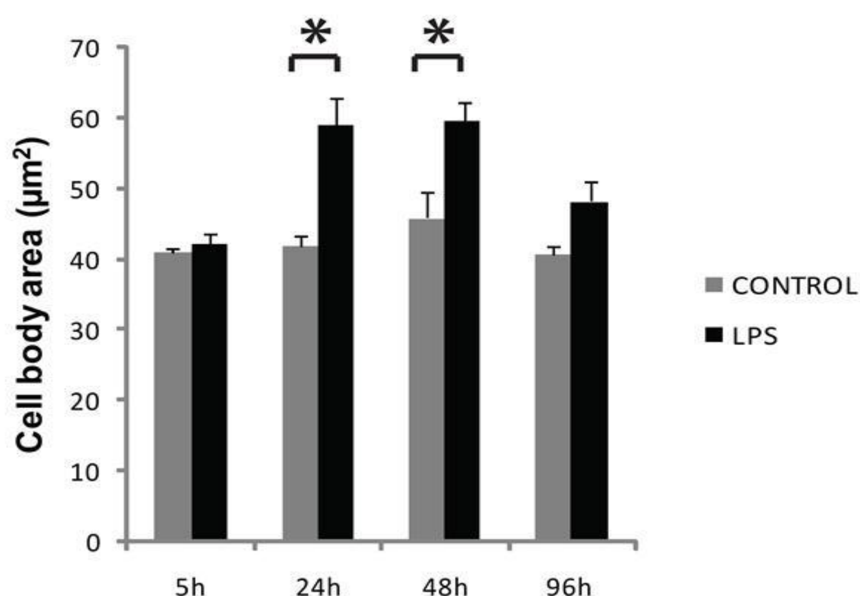

Figure 3-4. Average cell body area reveals hypertrophy at 24h and 48h and no significant difference at 5h and 96h (vertical axis: cell body area expressed in µm2; horizontal axis: time).

3.1.3 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 (see Table 3-8). An ANOVA analysis 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 ($F(3,16)=0.37$; $p=0.77$, see Table 3-9). Newman Keul's post-hoc analysis confirmed higher expression of CD11β was found in LPS-treated animals versus saline-treated animals ($p<0.01$) as shown in Table 3-10. LPS injected intraperitoneally increased the overall expression of CD11β as shown in Figure 3-5.

Microglia CD11β proportion				
	Mean (n=3)		Standard error	
	CONTROL	LPS	CONTROL	LPS
5h	0.487	0.534	0.032	0.032
24h	**0.487**	**0.665**	0.072	0.060
48h	**0.487**	**0.658**	0.099	0.017
96h	**0.487**	**0.578**	0.067	0.023

Table 3-8. Normalized proportion of microglial cells expressing CD11β. Microglia show an overall higher proportion of CD11β when treated with LPS.

Statistical analysis (ANOVA) of proportion of microglia expressing CD11β		
Effect	**F**	**p-level**
Treatment	8.987258	**0.008517**
Time	2.741545	0.077466
Treatment and time	0.375649	0.771764

Table 3-9. An ANOVA analysis revealed that the proportion of microglia expressing CD11β is increased when mice are treated with LPS respect to Control groups no effect of time or treatment by time interacition was found.

Post hoc analysis, main effect: Treatment		
	Control	LPS
Mean	0.4966	0.6116
Control		**0.0086**
LPS	**0.0086**	

Table 3-10. A post hoc test confirmed that the proportion of microglia expressing CD11β is increased when mice are treated with LPS respect to Control groups.

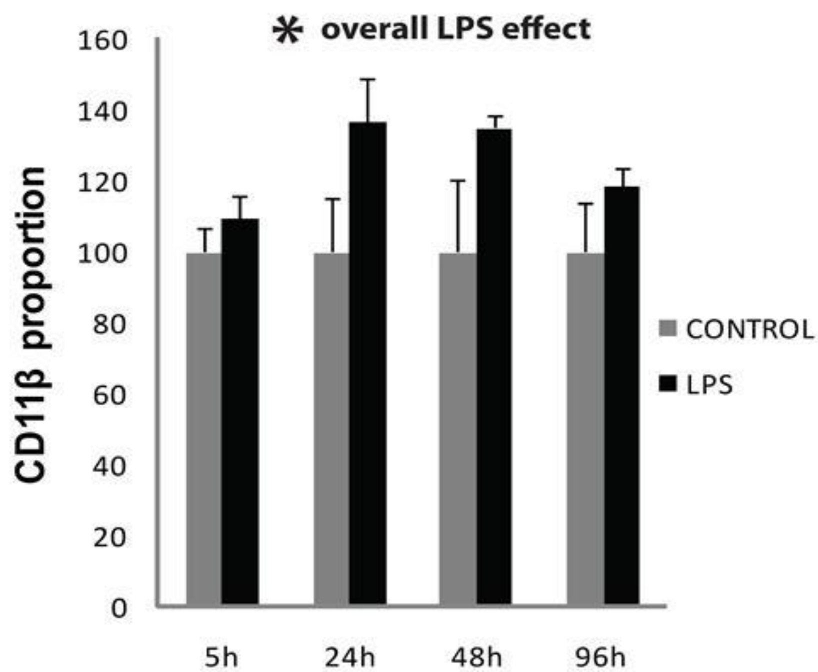

Figure 3-5. Average proportion of CD11β positive cells in the short term after LPS-saline administration showing an overall higher expression of CD11β in LPS treated mice (vertical axis: variation from control groups proportion of CD11β^{+} microglia).

3.2 Experiment 2: Long Term Effects of LPS on Microglia and Neurogenesis

3.2.1 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 (see Table 3-11). 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 Table 3-12). This data confirms that microglia did not present with an active phenotype at 1 week nor after up to 1 month after LPS administration (see Figure 3-6).

Microglia cell body area				
	Mean (n=3)		Standard error	
	CONTROL	LPS	CONTROL	LPS
1 Week	43.69	46.68	0.65	1.48
1 Month	41.29	42.20	2.61	1.19

Table 3-11. Microglia cell body areas and standard errors expressed in mm^2.

Statistical analysis (ANOVA) of microglia cell body area		
Effect	F	p-level
Treatment	1.399666	0.270743
Time	4.364287	0.070117
Treatment and time	0.40582	0.541895

Table 3-12. An ANOVA analysis of microglia cell body areas revealed no effect due to treatment time or interaction effect at 1 week and/or 1 month.

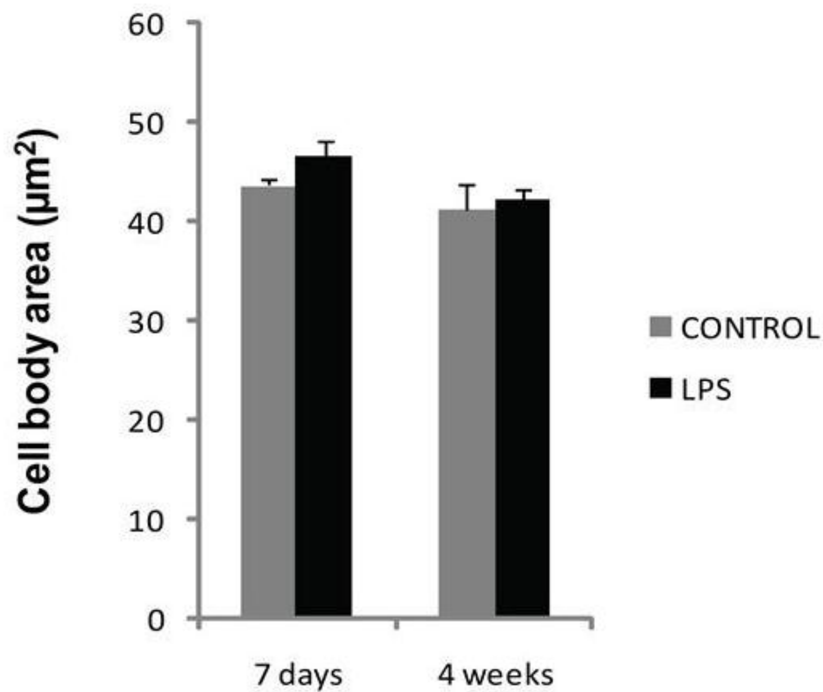

Figure 3-6. Cell body area expressed in µm^2 showing no difference between LPS and saline treated animals at 1 and 4 weeks.

3.2.2 Peripheral LPS Does Not Stimulate Phagocytic Activity in Microglia in the Long Term

All IBA-1$^+$ microglial cells expressed the phagocytic marker CD68 at low-level (see Figure 3-7). We therefore, confirmed that CD68 pixel intensities co-localized with IBA-1 were similar in the hippocampi of LPS-and saline-treated mice (see table 3-13). An ANOVA analysis confirmed that there was neither an effect of treatment ($F_{(1,8)}$=0.25; p=0.62) nor time after treatment ($F_{(1,8)}$=3.20; p=0.11) and these effects did not interact ($F_{(1,8)}$=3.20; p=0.11; see table 3-14). These data suggest that peripherally administered LPS did not stimulate phagocytosis in microglia 1 month after LPS administration as shown in Figure 3-8.

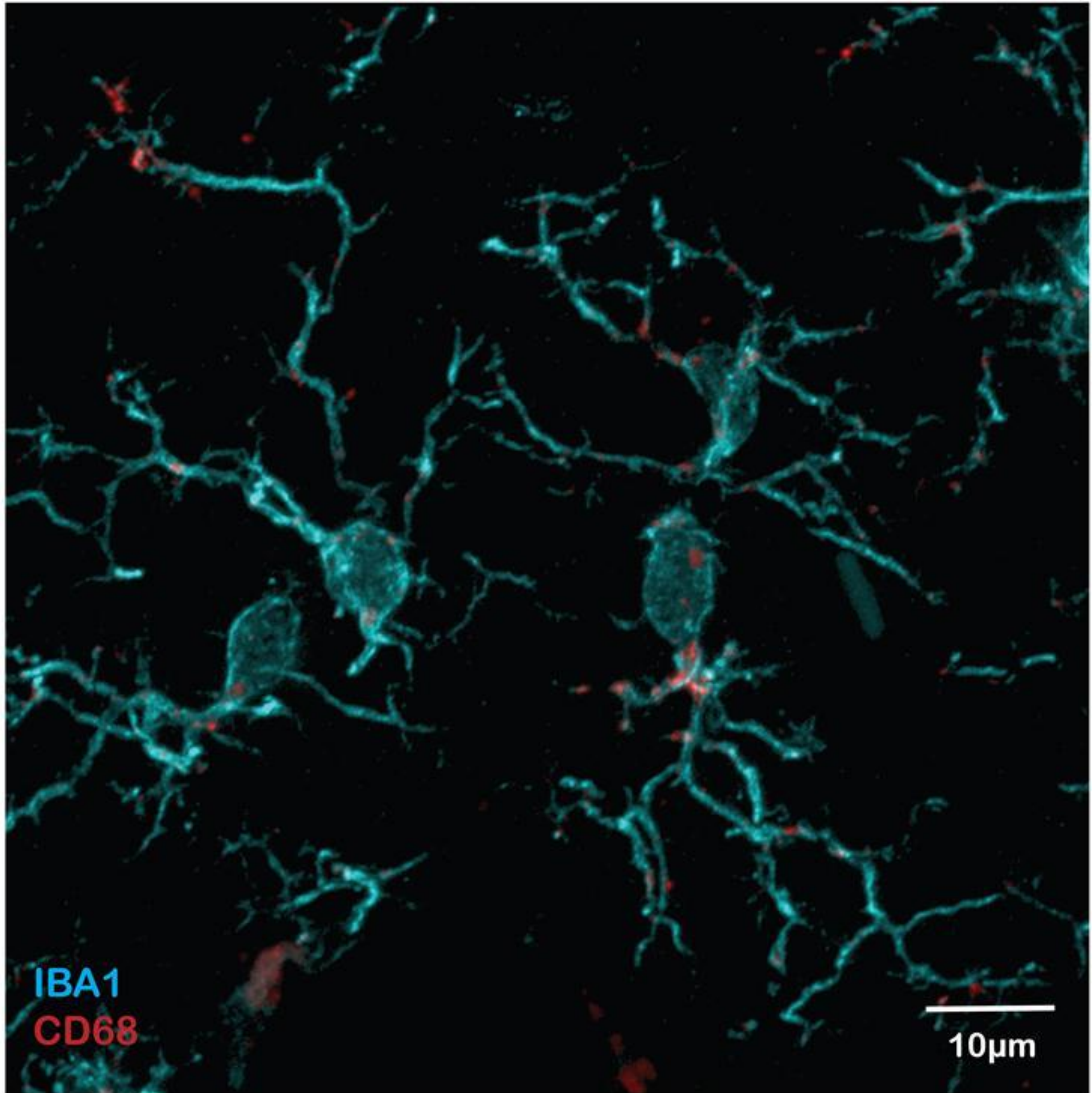

Figure 3-7. Low expression of CD68 is revealed in all microglia. Microglia identified with IBA-1(blue) and labeled with phagocytic marker CD68 (red). Figure shows a control animal killed 1 week after saline injection.

Intensity of CD68 pixels colocalized with IBA-1			
	Mouse 1	*Mouse 2*	*Mouse 3*
Control 1wk	104.0590	97.4169	98.5239
LPS 1 wk	97.4169	98.8892	93.3542
Control 1 M	96.665	95.50969	107.8253
LPS 1 M	107.4441	101.2862	109.7547

Table 3-13. Normalized pixel intensity values of CD68 (phagocytic activity marker) pixels colocalized with IBA-1 (microglial marker).

Statistical analysis (ANOVA) of intensity of CD68 colocalized pixels		
Effect	**F**	**p-level**
Treatment	0.256262	0.626352
Time	3.209008	0.111007
Treatment and time	3.209008	0.111007

Table 3-14. No statistically significant effect due to treatment, time or interaction effect is revealed analyzing pixel intensity of CD68 pixels co-localized with IBA-1, index of absence of phagocytic activity in the long term.

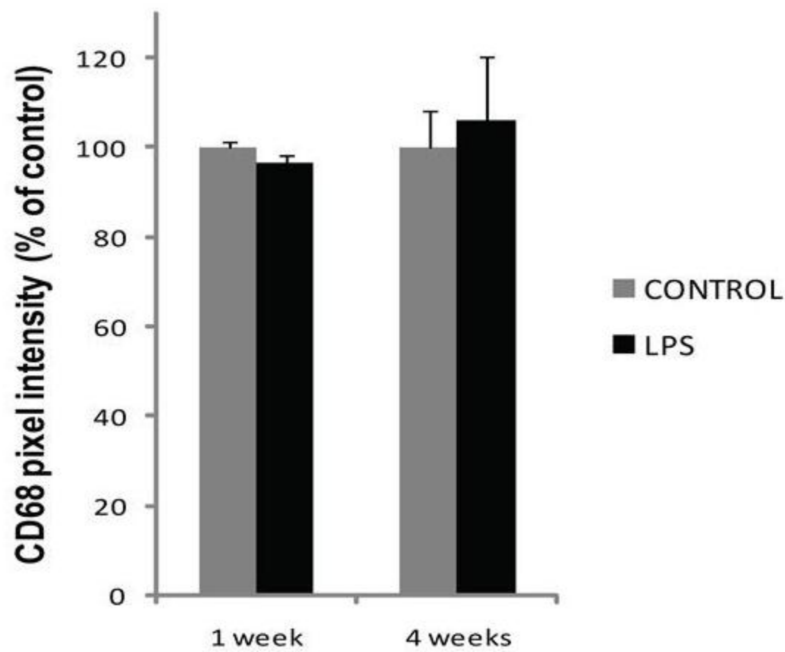

Figure 3-8. Expression of CD68 evaluated with pixel intensity revealing no effect of LPS at 1 and 4 weeks.

3.2.3 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 (see Table 3-15). An ANOVA analysis 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; see Table 3-16).These results show that intraperitoneal LPS does not induce a microglia active phenotype at 1 week and up to a month after LPS administration as shown in Figure 3-9.

Microglia CD11β proportion				
	Mean (n=3)		Standard error	
	CONTROL	LPS	CONTROL	LPS
7 days	0.487	0.544	0.059	0.070
3 weeks	0.487	0.484	0.077	0.083

Table 3-15. Normalized proportion of microglial cells expressing CD11β.

Statistical analysis (ANOVA) of proportion of microglia expressing CD11β		
Effect	**F**	**p-level**
Treatment	0.15139	0.707363
Time	4.172679	0.075356
Treatment and time	0.191603	0.673163

Table 3-16. ANOVA analysis reveals no effect of time, treatment or interaction effect in the proportion of $CD11\beta^{+}$ microglial cells in the long term.

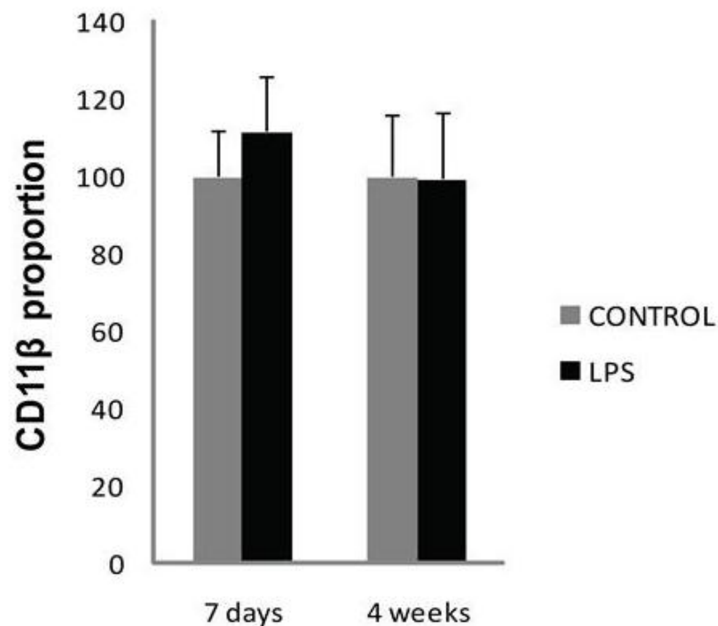

Figure 3-9. Proportion of CD11β positive cells express as variation from control, showing absence of effect due to LPS administration at 1 and 4 weeks.

3.2.4 Neuronal Differentiation is Transiently Compromised by Peripheral LPS

To test neural differentiation rate we evaluated the expression of BrdU, NeuN and DCX (see Figure 3-10) and calculated the proportion of new ($BrdU^{+}$) cells that expressed DCX and/or NeuN, respectively an early progenitor marker and a neuronal marker (see Table 3-17).

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 (see Table 3-18). 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 ($p>0.60$; see table 3-19). 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 3-11.

We can also evaluate as an average of 15% of new cells express DCX, index of progenitors in an early differentiation stage, meanwhile an average of 61% of new neurons express DCX and NeuN and are in a transition stage between neural committed progenitors and mature neurons (not considering the effect of LPS at 1 week where the proportion of cells co-expressing DCX and NeuN decreases to 41%), only 7% of new cells express just a mature neural marker NeuN.

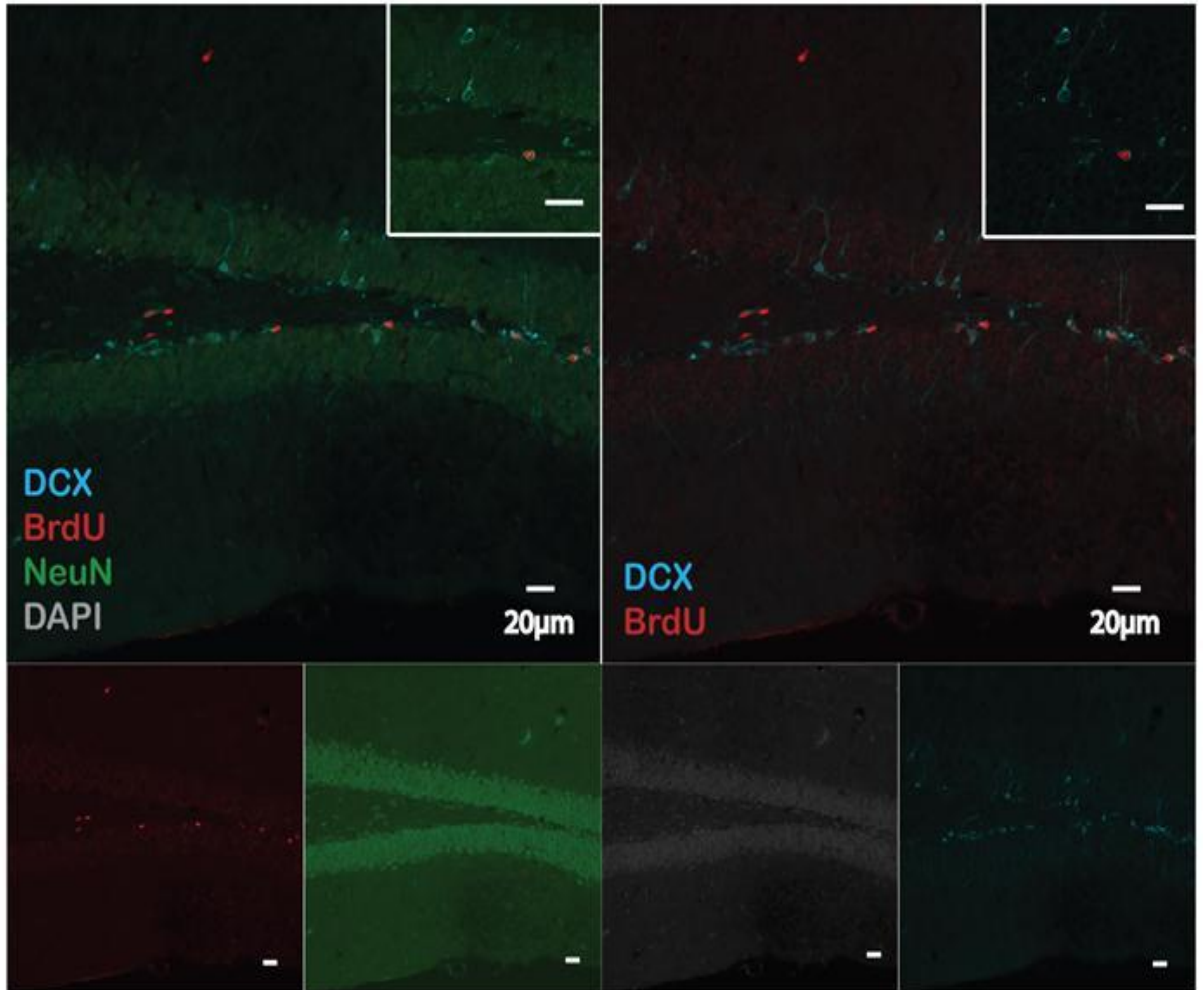

Figure 3-10. Analysis of progenitor phenotype, image from a mouse injected with BrdU one week after LPS-saline. A $BrdU^+/DCX^+/NeuN^+$ cell is shown at higher magnification. DCX a marker indicating an early stage of differentiation of neural progenitors into neurons is represented in light blue, BrdU a timidyne analog labeling new cells is colored in red, adult neural marker NeuN is colored green and cell nuclei (DAPI) in gray.

New cells phenotype								
		Average (n=3)				Standard error		
		DCX	DCX/NeuN	NeuN		DCX	DCX/NeuN	NeuN
1 week	Control	17.80	62.41	6.31		0.95	3.98	1.52
	LPS	17.63	41.50	7.43		6.91	9.85	0.85
2 week	Control	16.02	61.88	5.63		4.24	4.91	1.52
	LPS	15.42	60.08	8.35		1.08	2.65	0.54
3 week	Control	12.12	66.63	4.57		1.55	1.84	0.63
	LPS	12.93	62.12	7.30		0.74	2.12	0.22
4 week	Control	11.40	59.40	8.99		1.80	4.96	4.84
	LPS	16.93	56.63	8.72		4.51	2.94	0.96

Table 3-17. New cells identified with BrdU (thymidine analog marker) and proportion of markers co-expressed, in particular DCX and NeuN respectively early neural progenitor marker and an adult neural marker.

Statistical analysis (ANOVA) of proportion of new cells (BrdU positive cells)		
Effect	**F**	**p-level**
Treatment	3.948934	0.064298
Time	1.997736	0.154965
Phenotype	319.0469	**0.000000**
Treatment and Time	5.126803	**0.011264**
Treatment and Phenotype	3.037533	0.061965
Time and Phenotype	1.744405	0.142690
Time Treatment And Phenotype	0.834542	0.552354

Table 3-18. An ANOVA of new cells expression of early neural progenitor (DCX) and mature neural marker (NeuN) revealed an effect of phenotype and an interaction effect of treatment and time. The effect of phenotype means that considering all time point and group together the different phenotypes have a statistically significant difference, but the more important result is the interaction effect of time and treatment, that needs further investigation trough a post hoc test.

Post hoc analysis, main effect: Interaction Time x Treatment									
		5h	1wk	2wk	3wk	5h	1wk	2wk	3wk
		Control	Control	Control	Control	LPS	LPS	LPS	LPS
Mean		28.8433	27.8455	27.7733	26.5988	21.9588	27.9488	27.4522	27.4255
5h	Control		0.8050	0.9041	0.7833	**0.0090**	0.5791	0.8998	0.9415
1wk	Control	0.8050		0.9642	0.9300	**0.0189**	0.9487	0.9665	0.9932
2wk	Control	0.9041	0.9642		0.8780	**0.0150**	0.9933	0.8415	0.9737
3wk	Control	0.7833	0.9300	0.8780		**0.0098**	0.9520	0.8528	0.6079
5h	LPS	**0.0090**	**0.0189**	**0.0150**	**0.0098**		**0.0215**	**0.0148**	**0.0086**
1wk	LPS	0.5791	0.9487	0.9933	0.9520	**0.0215**		0.9889	0.9972
2wk	LPS	0.8998	0.9665	0.8415	0.8528	**0.0148**	0.9889		0.9868
3wk	LPS	0.9415	0.9932	0.9737	0.6079	**0.0086**	0.9972	0.9868	

Table 3-19. Post hoc analysis on Time by Treatment interaction revealed an effect at 5h, in particular 5h group appears to be the only group where LPS animals have a different proportion of differentiation cells, and this might be attributed to the reduction of new cells expressing DCX/NeuN when animals are injected with BrdU (thymidine analog) 5 hours after LPS injection. As expected no difference is present within different control groups .

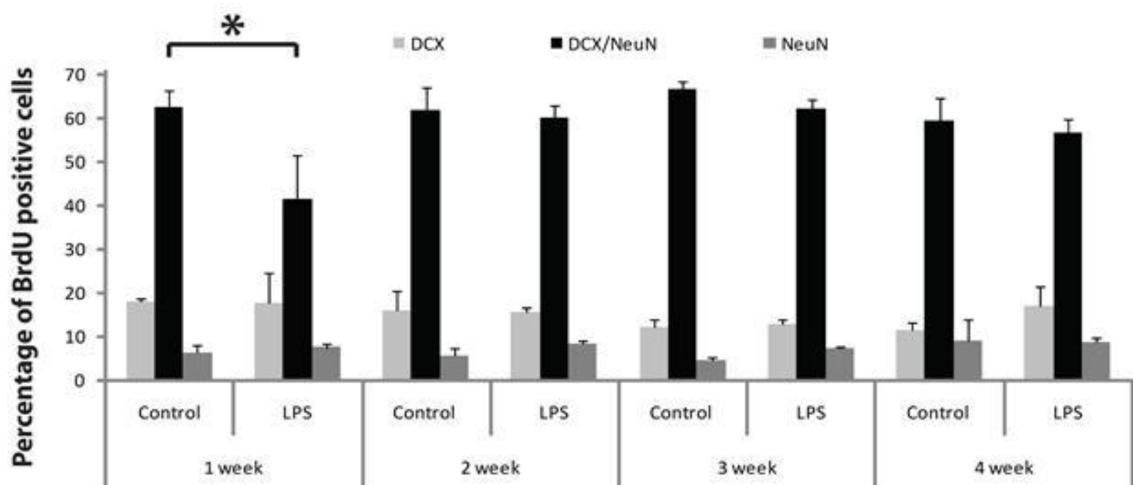

Figure 3-11. Analysis of progenitors phenotype revealed a reduction in BrdU/DCX/NeuN proportion of new cells in animals treated with BrdU 5h after LPS administration. No difference was revealed when BrdU is injected at 1 week, 2 weeks or 3 weeks after LPS-saline injection and animals are killed one week after BrdU (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 non-steroidal anti-inflammatory drug (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-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 non-steroidal anti-inflammatory drug (NSAID; i.e. minocycline) has 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 cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) and have shown to reduce microglia cytokine production as well as they might be reduce the blood brain barrier 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 these 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 contrasts other studies on rodents, 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 Professor Ormerod 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 Bachelors 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, USA to complete his Master of Science in Biomedical Engineering under the supervision of Dr. Brandi Ormerod.

Upon competition of his studies at both the Politecnico di Milano and the University of Florida he plans to pursue a career in the research field.