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PhD Thesis

“Inflammatory Stress and Adult Neurogenesis”

«Φλεγμονώδες Στρες και Ενήλικη Νευρογένεση»

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“Make a habit of two things: to help; or at least do no harm”

Hippocrates

“Healing is a matter of time, but it is sometimes also a matter of opportunity”

Hippocrates

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"There is no end to education. It is not that you read a book, pass an examination, and finish with education. The whole of life, from the moment you are born to the moment you die, is a process of learning."

Krishnamurti

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One should remember that the production of new knowledge acquires sacrifices, patience and mental strength. We should always keep in mind of two things: to help; or at least do no harm.

Ioannis Alexandros Gampierakis

Contents

Abstract

Inflammatory bowel disease (IBD), which comprises Crohn's disease (CD) and Ulcerative Colitis (UC), is a chronic gastrointestinal inflammatory condition with characteristic relapsing remitting phases. As the incidence of IBD is growing worldwide, new aspects of the pathophysiology of the disease and associated comorbidities are being recognized. Notably, increased incidence of psychiatric disorders and cognitive impairment has been observed in IBD patients that, together with the chronic inflammation, contribute to compromised quality of life. Emerging evidence suggests an intriguing interaction between the gut, brain and the immune systems, while any dysregulation in this communication is considered to affect the balance between central nervous system (CNS) homeostasis and neuropathology. Nevertheless, the impact of peripheral inflammatory processes, such as, those occurring in the context of IBD, on cognitive function and behavior and the specific immunological mechanisms involved remain incompletely understood.

Hippocampus is a key brain region implicated in cognitive processes, mood regulation and the pathophysiology of mood disorders. In addition, hippocampus is one of the brain regions with demonstrated active neurogenesis throughout life. The Neural Stem Cells (NSCs) niche in the Dentate Gyrus (DG) of the hippocampus is strongly associated with the modulation of cognitive processes, such as, pattern separation and cognitive flexibility, mediating the behavioral effects of stress and of depression therapy. As a result, dysregulation of hippocampal neurogenesis leads to memory and learning deficits and has been associated with the onset of depression and anxiety disorders.

A rapidly growing number of experimental and clinical findings suggest that systemic peripheral inflammation can modify synaptic plasticity and neuronal activity with associated, long-term behavioural changes. In fact, chronic neuroinflammation or peripheral inflammatory processes can disrupt the differentiation, maturation, migration and functional integration of new neurons in the hippocampal circuitry. Notably, traumatic brain injury or prolonged infusion of lipopolysaccharide (LPS), resulted in compromised integration of newborn neurons and decreased expression of the immediate early gene *Arc*, a marker of neuronal activity. Altered expression of Arc in mature and newborn neurons of the DG was highly correlated with the activation of microglia, while it also led to disruption of the regulated process of DG sparse coding as well as changes in memory function and mood. Interestingly, studies assessing hippocampus-dependent cognitive functions of IBD patients provided evidence of deficits in cognitive flexibility tasks, as evidenced by poor performance in the Subtle Cognitive Impairment test (SCIT). Moreover, structural changes in the hippocampal and para-hippocampal regions have been also revealed by functional magnetic resonance imaging (fMRI) studies in IBD patients, indicative of the association of this disease with hippocampal dysfunctions. Soluble factors produced in the periphery, even of high molecular weight, such as, inflammatory cytokines, can reach the brain, affect microglial responses and modulate cognition. Pertinent to intestinal inflammation, enhanced excitability and diminished long term-potentiation (LTP) and depression (LTD) were found in the

hippocampus of mice with colitis, together with signs of activated microglia and increased release of pro-inflammatory cytokines. Microglia and astrocytes play a pivotal role in the regulation of different steps in the neurogenesis process and, depending on their state of activation, can exert protective or inhibitory effects on the lineage of adult neural stem cells. Despite the increasing evidence on the effects of peripheral inflammatory processes in cognition and behavior, our understanding of the effects of acute and chronic intestinal inflammation on microglia and their potential link to hippocampal neurogenesis remains very limited. Seminal studies have documented that innate immune memory underlies long term microglia responses and the associated neuropathology. For example, systemic administration of repeated doses of lipopolysaccharide (LPS) induced an initial training of microglia, which was followed by the establishment of immune tolerance that lasted up to six months. Further, in response to a secondary inflammatory stimulus, such as Alzheimer's- or stroke-induced pathology, distinct effects of trained versus tolerant microglia have been shown, with exacerbation or alleviation of the manifestations of the disease by the former and the latter, respectively.

In the present study, we characterized the effects of experimental acute and chronic colitis, induced by administration of dextran sodium sulfate (DSS) in mice, on microglia activation and hippocampal neurogenesis. Our results showed that during acute DSS-induced colitis hippocampal microglia is activated, as evidenced by increased percentages of pro-inflammatory M1-like microglia and infiltration of inflammatory myeloid cells, along with elevated levels of pro-inflammatory cytokines, in the hippocampus. Microglia was induced in the neurogenic niche of the hippocampus along with increased astrogliosis. These findings were accompanied by enhanced neurogenesis, however with cell cycle deficits of proliferating neural progenitor cells. In contrast, microglia acquired an anti-inflammatory phenotype during chronic DSS colitis, as reflected by increased percentages of M2-like microglia, along with heightened levels of the anti-inflammatory cytokine IL-10, concomitant with decreased infiltration of myeloid cells and a marked reduction in pro-inflammatory cytokines. These findings were accompanied by deficits in the migration and integration of newborn neurons in the functional circuitry of the DG. Overall, our findings reveal that distinct immune pathways are induced during acute and chronic experimental colitis in the hippocampus, accompanied with changes in the neurogenic niche functions. These findings highlight potential underlying mechanisms that could exploited for the design of novel treatment modalities for better management of the off-site brain symptomatology found in IBD.

Περίληψη

Ο όρος Ιδιοπαθής Φλεγμονώδης Νόσος του Εντέρου (Inflammatory Bowel Disease-IBD) αναφέρεται σε χρόνιες, υποτροπιάζουσες διαταραχές του γαστρεντερικού σωλήνα, κυρίως στην Ελκώδη κολίτιδα (Ulcerative Colitis-UC) και τη Νόσο του Crohn (Crohn's disease-CD). Καθώς τα κρούσματα της νόσου αυξάνονται παγκοσμίως, όλο και περισσότερο αναγνωρίζεται η συνοσυρότητα που εμφανίζει με άλλες ασθένειες. Συγκεκριμένα, ασθενείς με κολίτιδα εμφανίζουν υψηλά ποσοστά ψυχιατρικών διαταραχών και γνωστικά ελλείμματα τα οποία σε συνδυασμό με την χρόνια φλεγμονώδη φύση της ασθένειας, συμβάλλουν στην κακή ποιότητα ζωής. Νέα δεδομένα δείχνουν ότι το έντερο, ο εγκέφαλος και το ανοσοποιητικό σύστημα επικοινωνούν αμφίδρομα, ενώ οποιαδήποτε διαταραχή αυτής της επικοινωνίας επηρεάζει την ισορροπία του κεντρικού νευρικού συστήματος που μπορεί να οδηγήσει σε διάφορες νευροπαθολογίες. Παρόλα αυτά, η επίδραση που έχουν οι περιφερικές φλεγμονές στην γνωστική λειτουργία και συμπεριφορά αλλά και οι ανοσολογικοί μηχανισμοί που σχετίζονται με αυτήν παραμένουν ακόμα υπό διερεύνηση.

Ο Ιππόκαμπος είναι μια περιοχή του εγκεφάλου που εμπλέκεται σε διάφορες γνωστικές λειτουργίες όπως είναι η μνήμη, η μάθηση και η ρύθμιση της διάθεσης, ενώ διαδραματίζει κεντρικό ρόλο στην παθοφυσιολογία των διαταραχών διάθεσης. Επιπρόσθετα, ο ιππόκαμπος είναι μια από τις περιοχές του εγκεφάλου στις οποίες υπάρχει νευρογένεση εφόρου ζωής. Τα νευρικά βλαστικά κύτταρα βρίσκονται τοπολογικά στην οδοντωτή έλικα (Dentate Gyrus-DG) του ιππόκαμπου και εμπλέκονται σε διάφορες γνωστικές λειτουργίες, όπως η διάκριση μοτίβου και η γνωστική ευελιξία, στο στρες αλλά και στην κατάθλιψη. Μια πληθώρα μελετών έχουν δείξει ότι η απορρύθμιση της νευρογένεσης στον ιππόκαμπο προκαλεί ελλείμματα στην μνήμη και στην μάθηση, ενώ έχει σχετιστεί με την ανάπτυξη κατάθλιψης και αγχωδών διαταραχών.

Νέες πειραματικές και κλινικές μελέτες έχουν δείξει ότι η συστημική περιφερική φλεγμονή μπορεί να τροποποιήσει την συναπτική πλαστικότητα και την νευρική δραστηριότητα με μακροπρόθεσμες αλλαγές στην συμπεριφορά. Συγκεκριμένα, η χρόνια νευροφλεγμονή ή η περιφερική φλεγμονή μπορεί να διαταράξει την διαφοροποίηση, ωρίμανση, μετανάστευση και λειτουργική ενσωμάτωση των νέων νευρώνων στο κύκλωμα του ιππόκαμπου. Ιδιαίτερα, ο τραυματισμός του εγκεφάλου ή η μακροχρόνια έγχυση βακτηριακού λιποπολυσακχαρίτη (lipopolysaccharide-LPS) προκαλεί αλλαγές στην ενσωμάτωση των νεών νευρώνων και μειωμένη έκφραση του πρώιμου γονιδίου *Arc*, που αποτελεί δείκτη νευρικής δραστηριότητας. Η τροποποιημένη έκφραση του Arc στους ώριμους και νέους νευρώνες της οδοντωτής έλικας του ιππόκαμπου σχετίζεται με την ενεργοποίηση της μικρογλοίας καθώς και με διαταραχές στην ενεργοποίηση του νευρικού κυκλώματος της οδοντωτής έλικας (DG). Επίσης, μελέτες σε ασθενείς με κολίτιδα στους οποίους μελέτησαν την απόκριση σε γνωστικές λειτουργίες που είναι εξαρτώμενες από την λειτουργία του ιππόκαμπου, έδειξαν ελλείματα στην γνωστική ευελιξία αυτών των ασθενών σε γνωστικά τεστ όπως το SCIT (Subtle Cognitive Impairment test). Επιπρόσθετα, δομικές αλλαγές σε ιπποκάμπιες και παρα-ιπποκάμπιες περιοχές έχουν ανιχνευθεί σε ασθενείς με κολίτιδα μέσω της λειτουργικής απεικόνισης μαγνητικού συντονισμού (fMRI), αποδεικνύοντας έτσι ότι η δομή αυτή

επηρεάζεται από την κολίτιδα. Παράγοντες στην περιφέρεια όπως είναι οι φλεγμονώδες κυτταροκίνες, μπορούν να φθάσουν στον εγκέφαλο, να επηρεάσουν την λειτουργία της μικρογλοίας και να τροποποιήσουν την γνωστική λειτουργία. Έχει δειχθεί ότι η πειραματική κολίτιδα αυξάνει την νευρική δραστηριότητα και μειώνει την συναπτική πλαστικότητα (long term-potentiation (LTP) and depression (LTD)) του ιπποκάμπου, ενώ ενεργοποιεί την μικρογλοία η οποία εκκρίνει περισσότερες προφλεγμονώδεις κυτταροκίνες. Η μικρογλοία και τα αστροκύτταρα διαδραματίζουν καθοριστικό ρόλο στην ρύθμιση των διαφόρων σταδίων της νευρογένεσης, και ανάλογα με το στάδιο ενεργοποίησης τους, μπορεί να επάγουν προστατευτικές ή κατασταλτικές επιδράσεις στα διάφορα στάδια των νευρικών βλαστικών κυττάρων. Παρόλη την αυξανόμενη βιβλιογραφία για τις επιδράσεις των περιφερικών φλεγμονών στην γνωστική λειτουργία και συμπεριφορά, οι επιδράσεις της οξείας και χρόνιας εντερικής φλεγμονής στην ενεργοποίηση της μικρογλοίας αλλά και στις επιδράσεις στην νευρογένεση του ιππόκαμπου παραμένουν ακόμα υπό διερεύνηση. Νέες μελέτες τείνουν προς το συμπέρασμα ότι η μικρογλοία μπορεί να αναπτύξει «μνήμη» σε ένα φλεγμονώδες ερέθισμα προερχόμενο είτε από την περιφέρεια είτε από το κεντρικό νευρικό σύστημα. Για παράδειγμα, η συστηματική χορήγηση του βακτηριακού λιποπολυσακχαρίτη (LPS) στην περιφέρεια επάγει μια αρχική ενεργοποίηση της μικρογλοίας (immune training) και μια μετέπειτα ανοχή (immune tolerance) στο φλεγμονώδες ερέθισμα η οποία διαρκεί για τουλάχιστον έξι μήνες. Η ύπαρξη ενός δεύτερου φλεγμονώδους ερεθίσματος, όπως είναι για παράδειγμα το εγκεφαλικό ή το Αλτζχάιμερ επάγει διακριτές επιδράσεις στην μικρογλοία ανάλογα με το αν υπάρχει προηγούμενη ενεργοποίηση ή ανοχή στο πρωτογενές φλεγμονώδες ερέθισμα. Στην περίπτωση της ενεργοποιημένης μικρογλοίας η παθολογία των δυο παραπάνω νόσων εντείνεται, ενώ στην περίπτωση της ανεκτικής μικρογλοίας η παθολογία των νόσων μειώνεται αντίστοιχα.

Στην παρούσα μελέτη, χαρακτηρίσαμε τις επιδράσεις της πειραματικής οξείας και χρόνιας κολίτιδας με την χρησιμοποίηση ενός πειραματικού μοντέλου χημικά επαγόμενης κολίτιδας που βασίζεται στην χορήγηση του δεξτρο-θεϊικού νατρίου (DSS) στο πόσιμο νερό, στην ενεργοποίηση της μικρογλοίας και στα διάφορα στάδια της ενήλικης νευρογένεσης του ιππόκαμπου. Τα αποτελέσματα μας έδειξαν ότι κατά την διάρκεια της οξείας κολίτιδας η μικρογλοία στην περιοχή του ιππόκαμπου ενεργοποιείται προς τον προφλεγμονώδη φαινότυπο (M1), ενώ υπάρχει αυξημένη διήθηση φλεγμονωδών μακροφάγων από την περιφέρεια και αύξηση των προ-φλεγμονώδων κυτταροκινών. Επίσης, η νευρογένεση στον ιππόκαμπο ήταν αυξημένη αλλά με αλλαγές στην κινητική του κυτταρικού κύκλου των διαιρούμενων νευρικών προγονικών βλαστικών κυττάρων. Αντίθετα, στην χρόνια φάσης της κολίτιδας, η μικρογλοία είναι ενεργοποιημένη προς τον αντιφλεγμονώδη φαινότυπο (M2), ενώ αυξημένα επίπεδα της ιντερλευκίνης 10 (IL-10) ανιχνεύονται τόσο στην περιφέρεια όσο και στην περιοχή του ιππόκαμπου. Παράλληλα, τα επίπεδα των προφλεγμονώδων κυτταροκινών καθώς και η διήθηση φλεγμονώδων μακροφάγων ήταν μειωμένα στην περιοχή του ιππόκαμπου. Κατά την διάρκεια της χρόνιας φάσης της κολίτιδας, οι νέοι νευρώνες παρουσίασαν τροποποιημένη μετανάστευση και λειτουργική ενσωμάτωση στο κύκλωμα του ιππόκαμπου. Ανακεφαλαιώνοντας, τα αποτελέσματα μας δείχνουν ότι κατά την διάρκεια της οξείας και χρόνιας κολίτιδας το ανοσοποιητικό σύστημα τόσο της περιφέρειας όσο και του εγκεφάλου ενεργοποιείται με έναν διακριτό τρόπο και με άμεσο

αντίκτυπο στα διάφορα στάδια της νευρογένεσης του ιππόκαμπου. Αυτά τα αποτελέσματα καταδεικνύουν την ύπαρξη μηχανισμών οι οποίοι μπορούν να αποτελέσουν την βάση για καινοτόμες θεραπείες για την στήριξη ασθενών με κολίτιδα που παρουσιάζουν συνοσυρότητα με διάφορες ψυχοπαθολογίες, όπως είναι η κατάθλιψη και οι αγχώδεις διαταραχές.

1. Introduction

Inflammation is widely recognized as a critical factor in the pathogenesis of numerous psychiatric disorders. Patients with systemic and chronic inflammatory diseases commonly experience complex disease behaviors, ranging from fatigue to mood disorders and cognitive impairments that arise from changes in brain function(1–3). These alterations in behaviour can greatly affect patient's quality of life. However, how peripheral inflammation is affecting the brain function and causes behavioural abnormalities, remains to a large extent unknown.

In the present study, we focused on unraveling the potential effects of experimental colitis in the process of adult hippocampal neurogenesis - a plasticity mechanism in the adult brain that is prone to environmental changes and inflammatory stress, as well as in the activation of the innate immune system of the hippocampus.

1.1 The Gut - Brain axis.

It is well established that gut physiology exerts a profound impact on brain activity and function. A great amount of evidence points to the role of gut brain axis in physiological and pathological conditions(4). The high rates of comorbidity between gastrointestinal and psychiatric diseases clearly indicate the reciprocal impact that gut homeostasis exerts in the cognitive and emotional centers of the brain. The brain communicates with the gut in a bidirectional way through neural, endocrine, metabolic and immune interaction roots(4). The gut-brain axis is considered as the mediator of the effects of both genetic and environmental factors on brain development and function and dysregulation of this axis is implicated in numerous psychiatric disorders including depression and anxiety(5).

Until recently, most of the research has focused on the contribution of gut brain axis in the regulation of digestive function and satiety, but now it is evident that gut brain axis has a key role in other aspects of physiology(5). Alterations in this axis has been implicated in gut inflammation, eating disorders, chronic abdominal and pain syndromes as well as in defects in stress response and behaviour. Epidemiological studies have shown an association between anxiety and gastrointestinal disorders including irritable bowel syndrome (IBS) and inflammatory bowel syndrome (IBD)(6,7). Further research should be done to explore the molecular mechanisms and pathways that mediate this communication in order to develop novel treatments to target psychiatric and gastrointestinal disorders.

Recent studies point to the role of gut microbiota in brain function even behaviour(5). The reciprocal impact of the microbiome and the development of psychopathologies is particularly interesting, given the fact that the microbiome can be altered by external factors such as antibiotics, diet, exposure to antimicrobials and disrupted sleep patterns.

1.2 Mechanisms and pathways that modulate gut brain axis in physiological and pathological conditions.

1.2.1 Neural pathways. The neural communication pathway is consisted of the central nervous system (CNS), the autonomic nervous system (ANS) and the enteric nervous system (ENS)(8).

The ANS consists of the parasympathetic and sympathetic nervous systems (SNS) and includes the vagus nerves (VNs), the sacral parasympathetic pelvic nerves, and the splanchnic nerves(4). The sympathetic system exerts an inhibitory influence on the gut via the release of neurotransmitters such as noradrenaline which decreases the intestinal motor function(6). Stress responses are mediated via the sympathetic system and the hypothalamic-pituitary-adrenal axis (HPA axis). The afferent signals of the ANS transmit to the brain via vagal (vagus nerve), spinal (dorsal ganglia root) and somatosensory afferents. These signals reach the nucleus tractus solitarious (NTS) in the brainstem and the periaqueductal gray (PG) and further synapse to the limbic system of the brain(6) which consists of the hippocampus, amygdala and limbic cortex (**Figure 1**). The limbic system is implicated in cognitive and emotional processes. The amygdala is implicated in fear and arousal responses, whereas hippocampus is implicated in memory and navigation processes(9). The limbic cortex is responsible for the regulation of olfaction and the integration of motor and sensory information. The communication process between the limbic and ANS underlie the link between behaviour and gut function in health and disease (eg. inflammatory bowel disease-IBD)(4).

The effector limb of the ENS integrates physiological responses such as gut motility and secretion and modulates the intestinal immune system mainly through a plethora of neurotransmitters such as acetylcholine (ACh), noradrenaline (NA), adrenaline, gamma-amino butyric acid (GABA), neuropeptides, such as substance P, neuropeptide Y, and opioids(10). The afferent limb of the ENS is comprised by sensory nerves that are responsible for gut reflexes and convey messages to the brain(10). These messages include information about the presence of pathogens, pro-inflammatory cytokines as well as signals about noxious stimuli (eg. gut distention)(10). CNS sent back responses or control messages to the ENS through efferent pathways, mainly via spinal (ventral motor root) or vagal efferent pathways. The ENS is divided into two neural plexuses: the myenteric and the sub-mucus plexus(10). The myenteric plexus, embedded into two layers of circular and longitudinal muscles, controls colon motility whereas the sub-mucus plexus lying on the submucosa layer of the digestive tube, directly controls GI blood flow and interacts with intraluminal and epithelial gut cells signaling(10).

1.2.2 Vagus nerve.

The vagus nerve provides the primary parasympathetic control of basic intestinal functions, with abundant innervation in the stomach, small intestine and appendix that decreases proximal to distal, terminating before the distal colon. The activation of vagus nerve leads to the release of acetylcholine (ACh) at the synaptic junction with secreting cells, intrinsic nervous fibers, and smooth muscles(8). ACh binds to nicotinic and muscarinic receptors and stimulates muscle contractions in the parasympathetic nervous system.

The gastrointestinal tract is highly innervated by vagal fibers that connect the central nervous system (CNS) with the intestinal immune system, which constitutes the vagus nerve a major component of the neuro-endocrine-immune axis(8). This axis is implicated in the co-ordination of neural, behavioural, and endocrine responses, important for the first line defense against inflammation. In response to pathogens and other injurious stimuli pro-inflammatory cytokines are produced by activated macrophages, dendritic cells and other cells in the mucosa. Pro-inflammatory cytokines like the tumor-necrosis factor-alpha (TNF-α), prostaglandins and interferons are important mediators of local and systemic inflammation. It is known that the dorsal vagal complex which comprises the sensory nuclei of the solitary tract, the area postrema, and the dorsal motor nucleus of the vagus, responds to increased pro-inflammatory cytokines like TNF-α by altering the motor activity of the vagus nerve. The vagus nerve exert anti-inflammatory properties(11,12). The anti-inflammatory capacities of the vagus nerve are mediated through three different pathways:

1. The first pathway is through the activation and regulation of the hypothalamus–pituitary–adrenal (HPA) axis. The vagal afferent pathways are involved in the activation/regulation of the HPA axis which is the endocrine core of the stress system. Pro-inflammatory cytokines activate HPA axis through secretion of the corticotropin-releasing factor (CRH) from the hypothalamus. The CRH release stimulates adrenocorticotropic hormone (ACTH) secretion from pituitary gland. This stimulation, in turn, leads to cortisol release from the adrenal glands. Cortisol is a major stress hormone that affects many human organs, including the brain, bones, muscles, and body fat. Furthermore, cortisol exerts immunosuppressive effects on macrophages, suppressing the production of pro-inflammatory cytokines(12).
2. The second pathway is the splenic sympathetic anti-inflammatory pathway. The vagus nerve stimulates the splenic sympathetic nerve which results in the release of norepinephrine (NE). NE is released in the distal end of the splenic nerve and is linked to the β2 adrenergic receptor of splenic lymphocytes which in turn release ACh. ACh inhibits the release of TNF-α by spleen macrophages through a7-nicotinic ACh receptors(8,12,13).
3. The third pathway is the cholinergic anti-inflammatory pathway (CAIP). This pathway is mediated through vagal efferent fibers that synapse onto enteric neurons, which in turn release ACh at the synaptic junction with macrophages. ACh binds to α-7-nicotinic ACh receptors of those macrophages and inhibit the action of TNF-α. The above pathway has some unique properties, such as, high speed of neural conductance, which enables an immediate modulatory input to the affected region of inflammation(8,12,13). Therefore, the CAIP plays a crucial role in the intestinal immune response and homeostasis and constitutes a highly promising target for the development of novel treatments for inflammatory diseases related to the gut immune system like IBD.

The vagus nerve is considered as an inflammatory reflex nerve(14). The existence of injury or pathogens activates immune system which in turn release pro-inflammatory cytokines. These cytokines activate sensory fibers that ascend in the vagus nerve to synapse in the nucleus tractus solitarius(14). Increased efferent signals in the vagus nerve suppress peripheral cytokine release through macrophage nicotinic receptors and the CAIP. It has been

shown that experimental activation of the CAIP by direct electrical stimulation of the efferent vagus nerve inhibits the synthesis of TNF-α in liver, spleen, and heart, and attenuates serum concentrations of TNF-α(14).

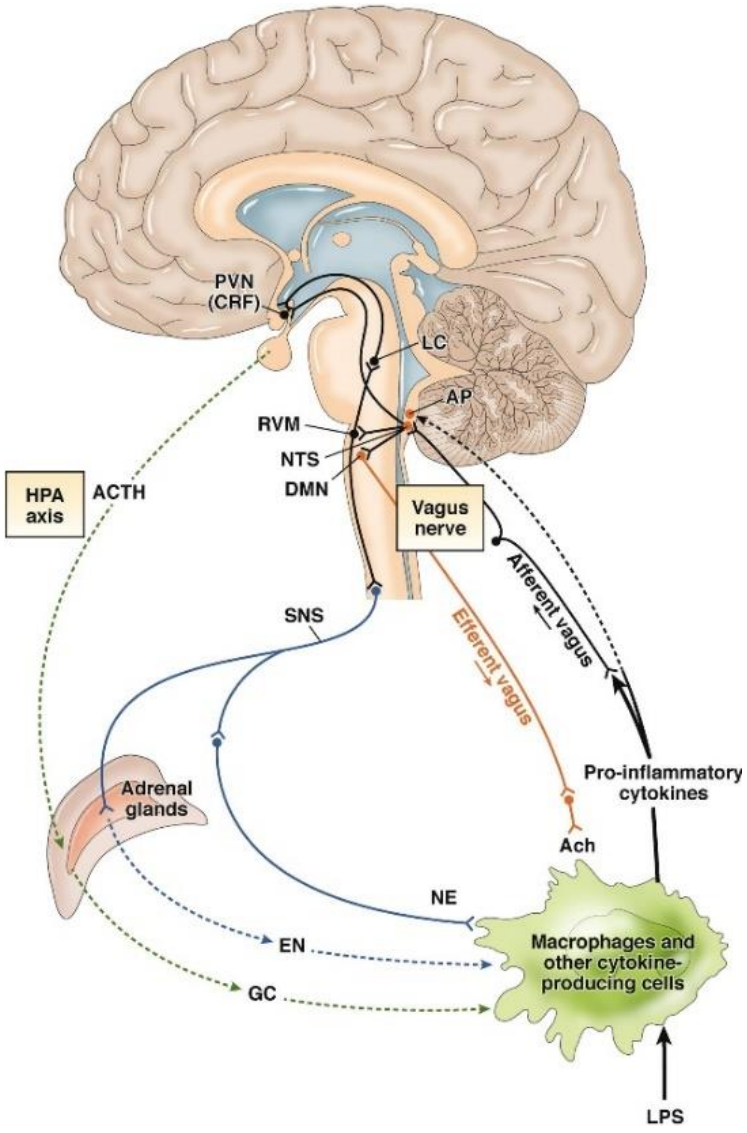

Figure 1. ***Neuroanatomical pathways of brain-gut axis.*** *The brain communicates with the gut through the autonomic nervous system (ANS) and the circumventricular organs. The ANS consists of the parasympathetic and sympathetic nervous system (SNS), which include the vagus nerves (VNs), the sacral parasympathetic pelvic nerves, and the splanchnic nerves. Visceral information is transmitted through ANS afferents and reaches CNS at 2 different centers: 1) the nucleus tractus solitaries (NTS) which receives VN afferents and 2) the thoracolumbar and sacral spinal cord, receiving splanchnic and pelvic nerves. The circumventricular organs, located outside the blood brain barrier (BBB), are sensitive to circulating pro-inflammatory cytokines and modulate the activity of neighboring neurons that in turn stimulate the hypothalamic-pituitary-adrenal (HPA) axis, that subsequently suppress mucosal inflammation via glucocorticoids (GC). A third pathway of brain gut communication is the cholinergic anti-inflammatory pathway (CAIP). This pathway is mediated through vagal efferent fibers that synapse onto enteric neurons, which in turn release acetylcholine (Ach) at the synaptic junction with macrophages. ACh binds to α-7-nicotinic ACh receptors of activated macrophages (e.g. LPS stimulation) to inhibit the action of pro-inflammatory cytokines such as TNF-α. This figure was adapted from Bonaz and Berstein, Gastroenterology, 2013; 144:36-49*

1.2.3 Gut microbiota.

A growing body of literature delineate the gut microbiota as a key regulator player of the CNS activity. The human gastrointestinal tract is inhabited by 1 x 10^{13} to 1 x 10^{14} microorganisms-more than 10 times of the number of human cells in our bodies and containing 150 times as many genes as our genome(9). The estimated number of species in the gut microbiota varies greatly, but it is generally accepted that the adult microbiota consists of more than 1.000 species and more than 7.000 strains(15,16). *Bacteroidetes* and *Firmicutes* are the two predominant bacterial phylotypes in the human microbiota. On the contrary, *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla present in relatively low abundance(15,16).

Changes in the composition of bacterial population in the GI have been associated with a wide array of pathological conditions including neurological and neurodevelopmental disorders such as multiple sclerosis, autism, depression, schizophrenia and neurodegenerative disorders such a Parkinson's disease(5,9). Intestinal bacteria may exert direct effects on host processes through the production of signaling molecules that interact with CNS, including hormones, and neurotransmitters such as monoamines and GABA(5). Profound changes in the composition of gut microbiota affect the levels of some of these molecules along with those of growth factors such as the brain derived neurotrophic factor (BDNF) in the brain, creating the possibility for significant functional alterations. The gut microbiome plays a significant role in modulating the release of a variety of gut peptides such as leptin and neuropeptide Y from the endocrine cells of the GI(9). These gut peptides can act on the function of CNS and play significant role in regulating circadian rhythms, anxiety levels and behaviour (**Figure 2**).

Gut bacteria modulate various host metabolic reactions and are responsible for the production of metabolites such as bile acids, choline and short-chain fatty acids (SCFAs) that are essential for the host health. SCFAs are known to have neuroactive properties and exhibit potent anti-oxidant and anti-inflammatory properties(17,18). SCFAs are produced by complex carbohydrates where they fermented in the colon by gut microorganisms into n-butyrate, acetate and proprionate(17). Moreover, the presence of SCFA-producing bacteria in the colon has been shown to strengthen the blood brain barrier (BBB) by increasing the expression of tight junction proteins(19). The aforementioned metabolites have been shown to alter host gene expression in the brain, providing additional evidence for the microbiota to influence the activity of the CNS(9). Interestingly, many of the effects of the gut microbiota on brain function has shown to be dependent on vagal activation(8). Moreover, activation of the vagus nerve has been shown to have marked anti-inflammatory capacity, protecting against microbial-induced sepsis in a nicotininc-acetylcholine receptor a7 subunit-dependent manner (**Figure 2**).

1.2.4 Immune activation.

It is well established that gut microbiota have direct effects on the immune system. Under normal, healthy conditions, mucus, a tight barrier of the epithelial cells confine most microbes to the intestinal lumen or the epithelial surface(20). The existence of microbes in the mucus layer stimulates the host intestinal immune system which predominantly promote

tolerance of the commensal microbes maintaining the barrier integrity without causing inflammation(20,21). On the contrary, the existence of an inflammatory condition due to damage of the intestinal barrier, the introduction of aggressive pathogens or the existence of substances that provoke strong immune reactions can accelerate the production of pro-inflammatory cytokines and reactive oxygen species which in turn through the bloodstream reach the CNS and affect its function(21). Systemic inflammation can directly alter BBB permeability. Existing evidence points to the role of inflammatory molecules such as cytokines, reactive oxygen species, matrix metalloproteases, and mediators of angiogenesis, with BBB disruption(22). In addition, a positive feedback loop exists, involving the pro-inflammatory cytokine IL-6 in conjunction with neuro-immune reflex circuits, increasing BBB permeability and the access of peripheral T cells in the brain. BBB permeability can significantly alter the immune responses to CNS antigens and compromise brain protection against potentially inflammatory substances(22). It is increasingly recognized that immune cells communicate with neurons directly by modulating neuronal activity via the release of neurotransmitters and cytokines (**Figure 2**).

1.2.5 HPA axis and the enteroendocrine system.

The humoral pathway of the gut-brain axis consists of the hypothalamic pituitary-adrenal axis (HPA axis) and the enteroendocrine system(4,23,24). The most well studied effects of intestinal inflammation on the CNS involve the hyper-reactivation of the HPA axis and imbalances in serotonergic activity(23). These changes have been associated with the manifestation of "sickness behaviour" as well as anxiety and depression(9,23). In addition, these psychological conditions are frequently observed as comorbidities in individuals with diseases characterized by persistent intestinal inflammation, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)(23,24). The enteroendocrine system contains the enteroendocrine cells which produces neuroactive hormones and molecules (such as ghrelin and cholecystokinin, 5- hydroxytryptamine,) and regulate appetite and a broad range of gut and brain functions(25) (**Figure 2**).

1.2.6 Tryptophan metabolism.

Tryptophan is one of the most essential amino acids and is the precursor to many biologically active agents, including the neurotransmitter serotonin(26). A growing body of evidence points to the role of the kynurenine arm of the tryptophan metabolic pathway, while dysregulation of this pathway has been implicated in the development of many disorders in the brain and GI(27). The presence of intestinal inflammation upregulates the activity of the indoleamine-2,3-dioxygenase (IDO) enzyme which convert tryptophan to kynurenine(27). Plasma levels of kynurenine, kynurenic acid, 3-hydroxykynurenine, and xanthurenic acid, are higher in IBD patients(27). The kynurenine is transported across the BBB into the brain, where it can further be metabolized in perivascular macrophages, microglia and astrocytes to generate neuroactive compounds(28). The decreased plasma levels of available tryptophan are involved in in the onset of the physio-somatic symptoms of depression and neurotoxic processes, including defective neuroplasticity and neurogenesis(28) (**Figure 2**).

1.2.7 Intestinal permeability.

Systemic and chronic intestinal inflammation may increase the permeability of the GI.

Injury in the epithelium or invasion of pathogens can cause temporary but substantial defects in the epithelial barrier(29). Intestinal microbiota regulates the expression of tight junctions in the intestinal epithelium and many pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α negatively impact the expression of tight junctions, thus increasing the barrier permeability(29). The later, facilitate the recruitment of immune cells and components from the circulation to sites of inflammatory insult. A weakened and permeable epithelium could lead to the influx of microbial products into the bloodstream resulting in the acceleration of immune activation. Sustained permeability could lead to the release of microbial components such as lipopolysaccharide (LPS) into the systemic circulation. These sites are highly immunogenic and trigger systemic inflammatory responses. This condition is called the "leaky gut syndrome" and characterizes a plethora of GI pathologies like IBS, IBD, metabolic syndrome and diabetes(30). Intestinal permeability has been implicated in pathological conditions of the CNS such as autism, schizophrenia, multiple sclerosis, depression, anxiety and post-traumatic stress disorder(9). Recent years have witnessed the rise of the gut microbiota as a major topic of research interest in biology. Studies are revealing how variations and changes in the composition of the gut microbiota influence normal physiology and contribute to diseases ranging from inflammation to obesity(30). Accumulating data now indicate that the gut microbiota also communicate with the CNS--possibly through neural, endocrine and immune pathways--and thereby influences brain function and behaviour(5). Studies in germ-free animals and in animals exposed to pathogenic bacterial infections, probiotic bacteria or antibiotic drugs suggest a role of the gut microbiota in the regulation of anxiety, mood, cognition and pain(31). Thus, the emerging concept of a microbiota-gut-brain axis suggests that modulation of the gut microbiota may be a tractable strategy for developing novel therapeutics for complex CNS disorders. Especially, the increased levels of LPS can compromise both active and passive BBB mechanisms, rendering the CNS vulnerable to neurotoxic substances and activated immune cells from the periphery(9). This in turn may activate microglia- the resident immune cells of the brain- to release pro-inflammatory cytokines which is known to alter CNS activity and function(20). Pro-inflammatory cytokines and oxidative stress have been causally linked to neuron death and altered synaptic plasticity, with neuroinflammation to be considered key player in numerous neurodegenerative diseases(32) (**Figure 2**).

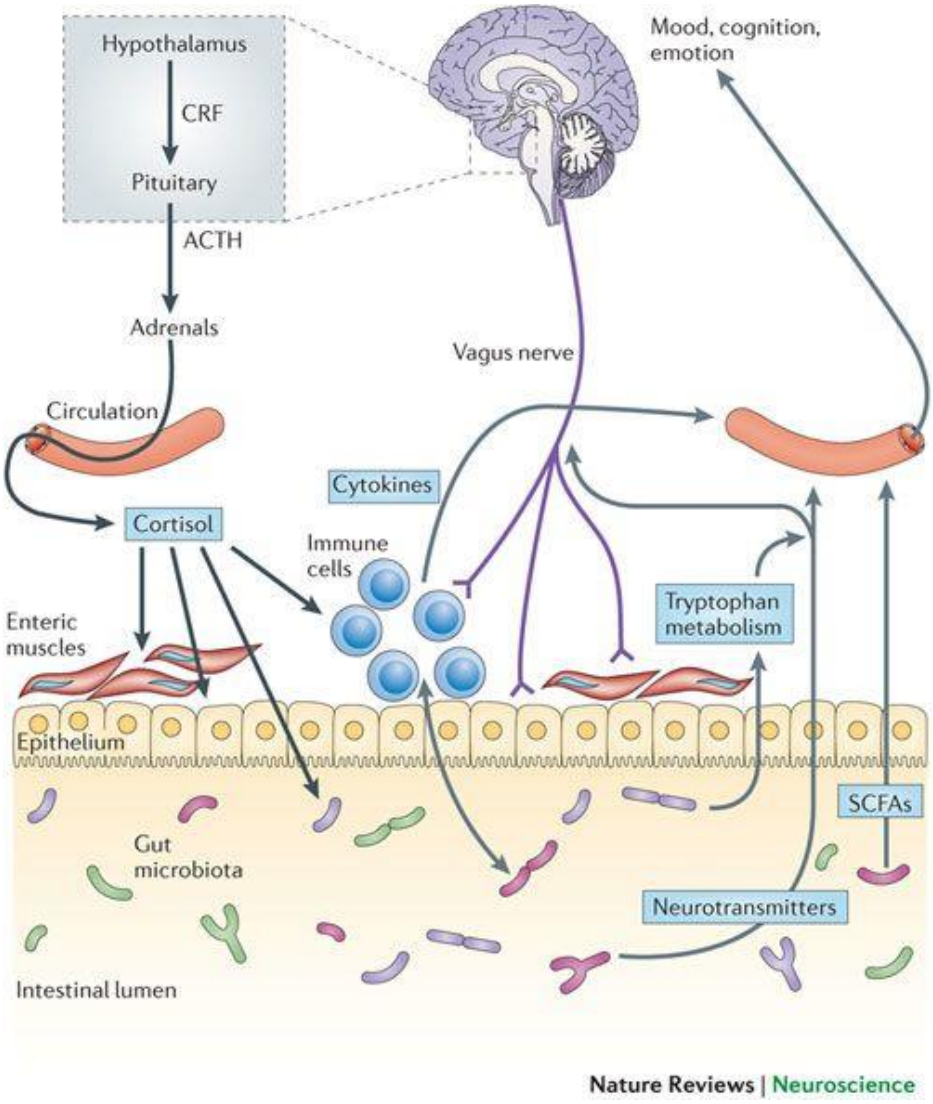

Figure 2. Pathways involved in the bidirectional communication of the gut and the brain. *Multiple direct and indirect gut-brain pathways exist. They include endocrine (cortisol), immune (cytokines) and neural (vagus and enteric nervous system) pathways. The hypothalamus–pituitary– adrenal (HPA) axis regulates glucocorticoids secretion which affect immune cells (including cytokine secretion) both locally in the gut and systemically. Glucocorticoids can also alter gut permeability and barrier function, and change gut microbiota composition. Gut microbiota can alter the levels of circulating cytokines, and this can have a marked effect on brain function. Both the vagus nerve and modulation of systemic tryptophan levels are strongly implicated in relaying the influence of the gut microbiota to the brain. In addition, short-chain fatty acids (SCFAs) functions as neuroactive metabolites, thus modulating brain function and behaviour. ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone. This figure was adapted from Cryan and Dinan, Nature Reviews, Neuroscience, 2012; Volume 13; 701-712*

1.3 Inflammatory Bowel Disease (IBD).

Inflammatory bowel disease (IBD), which comprise Crohn's disease (CD) and Ulcerative colitis (UC), is a chronic relapsing disorder of the gastrointestinal tract that is characterized pathologically by intestinal inflammation and epithelial injury(33). Both disorders have a major impact on an individual's quality of life and their ability to work. The estimated prevalence of IBD is 200 per 100,000 people(6). IBD is characterized by abdominal pain with frequent, loose stools and malaise. In the acute phase, bloody diarrhea is seen and in some cases might be indistinguishable from infectious enterocolitis(34). The diagnosis of IBD is complex and relies on a chronic remitting and relapsing history along with endoscopic and histological investigations showing chronic inflammation(34).

Studies in recent years have identified a major role of both genetic and environmental factors in the pathogenesis of IBD(16,35). A combination of genetic and environmental factors affects the proper epithelial barrier function resulting in the translocation of microbial antigens. Subsequently, the intestinal immune system is activated causing acute inflammation(36). If acute intestinal inflammation fails to be resolved, chronic intestinal inflammation develops, due to the uncontrolled activation of the mucosal immune system. More specifically, the mucosal immune cells which include macrophages, T-cells and the innate lymphoid cells (ILCs) seems to respond to microbial products or antigens from the gut microbiota by producing pro-inflammatory cytokines that further promote chronic inflammation of the GI(37) (**Figure 3**).

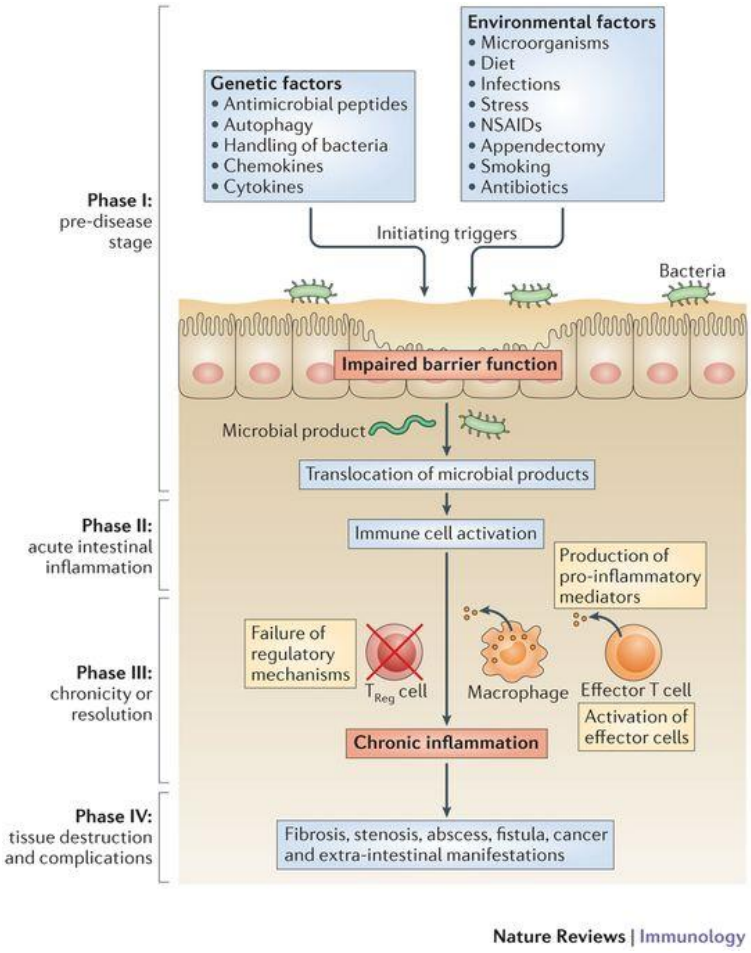

Figure 3. Pathogenesis of IBD. *Phase I: Genetic and environmental factors are initiating triggers for impaired barrier function in the intestinal mucosa. Impaired barrier function induces the translocation of commensal bacteria and microbial products from the gut lumen into the bowel wall, leading to immune cell activation and cytokine production. Phase II and III: Acute intestinal inflammation develops and if it is not resolved by anti-inflammatory mechanisms through suppression of pro-inflammatory immune responses, chronic intestinal inflammation is developed. Phase IV: Chronic inflammation causes tissue destruction and complications, mainly driven by mucosal cytokine responses. DC: dendritic cells; IBD: Inflammatory Bowel Disease; NSAIDS: non-steroidal anti-inflammatory drugs; T_{reg} cell, regulatory T cell. This figure was adapted by Neurath, Nature Reviews Immunology, 2014*

Genome wide association studies (GWAS) have identified a number of 163 gene loci related to IBD, of which 110 are associated with CD and UC, 30 are CD specific and 23 are UC specific(38). At 2001, the first susceptibility gene for CD was identified; the *NOD2* gene (nucleotide-binding oligomerization domain containing 2 gene)(39). *NOD*2 gene encodes for an intracellular receptor that recognizes the muramyl dipeptide (MDP) which is a conserved motif present in the peptidoglycan from both Gram-positive and negative bacteria(39). Stimulation of the MDP induces autophagy in order to control bacterial replication and antigen presentation(40). Thus, it is important for the modulation of both the

innate and adaptive immune system. *NOD2* gene is implicated in distinct MDP-independent pathways including the pathways that regulate the T-cell response(40). Further GWAS association studies have implicated 2 autophagy-related genes in IBD pathology: *ATG16L1* and *IRGM*(41). The coding mutation T300A of *ATG16L1* gene is associated with an increased risk for CD(42). IRGM belongs to the p47 immunity related GTPase family(41). CD-associated polymorphisms in *IRGM* gene lead to reduced protein expression(41). It has been shown that epithelial and dendritic cells in the mucosa containing *ATG16L1* and *NOD2* variants show deficits in antibacterial autophagy(43). Furthermore, immune-related genes have recently been associated with IBD. One of those genes is the *IL23R* gene which encodes a subunit for the receptor for the pro-inflammatory cytokine interleukin 23 (IL-23)(44). IL-23 is involved in the generation of Th17 cells. Also, susceptibility gene loci *IL23R*, *IL12B*, *JAK2* and *STAT3* have identified in the pathogenesis of both CD and UC(45). Defects in the function of *IL-10* gene has also been associated with CD and UC and IL-$10^{-/-}$ mice have been generated to study the pathophysiology of colitis(46). Other susceptibility genes that regulate immune function include *CARD9*, *IL1R2*, *REL*, *SMAD3* and *PRDM1*.

Environmental factors are important variables for the pathogenesis of IBD. Smoking, stress, air pollution, vitamin D deficiency, anti-inflammatory drugs and antibiotics are among the environmental factors that in relation with a genetic susceptible individual could contribute to the development of either CD or UC(47). Smoking increases the risk of CD and it has been associated with a higher rate of postoperative disease(48). Stress is considered as one of the most important variables in the development of IBD. Individuals with lower levels of stress exhibit reduced risk to develop IBD, while anti-depressant therapy benefits a significant proportion of IBD patients(49). Air pollution has recently been considered as a variable that may contribute to the development of IBD(50). Recent studies have found that air pollution is associated with an upregulation in circulating polymorphonuclear leukocytes and plasma cytokines(50). Another study suggests that high levels of NO_2 and SO_2 correlate with increased risk of CD and UC(50). Also, pollutant emission has been linked to increased rates of hospitalizations for IBD patients further suggesting that the quality of the air constitute an important factor in the disease progression(50). Recent studies have shown that vitamin D deficiency is a common trend among IBD patients and is associated with an increased risk for IBS(51). Anti-inflammatory drugs (NSAIDs) when used chronically are associated with an increased risk of CD and UC(52). Antibiotics through their action in the microbiome affect a variety of physiological actions and chronic administration alters the integrity of the mucus layer(53). The microbiome of IBD patient's lack of some species that are present in healthy individuals. More specifically, the species *Furmicutes* and *Bacteroidetes* are downregulated and species like enterobacteria are upregulated in CD patients; meanwhile, a reduction in *Clostridium spp*. and an increase in *Escherichia coli* have been reported in UC patients(53). An altered microbiome affects the function of the intestinal immune system leading to inflammatory exacerbations as well as imbalances in the innate and adaptive immune system response. More specifically, CD is driven by Th1 response and UC with non-conventional Th2 response. Most immunological studies are now focusing in the role of the innate immune system in barrier integrity, microbial sensing and autophagy in experimental models of colitis(34).

The intestinal barrier consists of intestinal epithelial cells (IECs) and innate immune cells that together maintain the balance between luminal contents and the mucosa. The importance of the intestinal barrier is well established in IBD patients, which present abnormal permeability and alterations in the expression of the junctional protein epithelial cadherin(54). Of note, transcription factors implicated in epithelial regeneration such as HNF4A (hepatocyte factor 4a) and NKX2-3 (NK2 homeobox 3) are associated with IBD(55). Apart from the role of IECs in the maintenance of the equilibrium of lumen contents and the mucosa, IECs comprise a heterologous population of cells including enterocytes, goblet cells, neuroendocrine cells, Paneth cells, and M cells(33). Goblet cells produce the mucus matrix covering the epithelium, essential to both mucosal defense and repair. In murine models, knockout of Muc2 (mucin 2), a major goblet cell derived secretory mucin, results in spontaneous colitis(56). Resistin-like molecule b (RELMb), another goblet cell specific protein is implicated in the communication between the immune system by directing Th2 cell immunity and delivering luminal antigens to tolerogenic sets of dendritic cells(57). Paneth cells reside at the base of small intestinal crypts, and are responsible for crypt homeostasis by maintaining the intestinal stem cell niche, and secretion of antimicrobial effector cells that control the balance between the microbiome and mucosa(58). They have been identified several genes implicated in IBD pathology associated with Paneth cells function, most notably NOD2 and autophagy(40). NOD2 is expressed from Paneth cells, dendritic cells, macrophages and absorptive IECs. As we discussed above, NOD2 variants are associated with lower levels of defensins in Paneth cells leading to impaired antimicrobial function. Patients with NOD2 and ATG16L1 mutations, present with aberrant secretory apparatus of Paneth cells, resulting in defects in antibacterial autophagy(59). In dendritic cells, defects in these genes induce impairments in the antigen presentation to T cells.

In the healthy gut, intestinal macrophages exist in a state of hyporesponsiveness, showing increased proliferation and chemotactic activity in response to cytokines and microbial agents, while retaining the phagocytic and bactericidal function(34). These macrophages produce anti-inflammatory cytokines to induce T-cell differentiation and restrain Th1 and Th17 cell responses(34). Patients with Crohn’s disease exhibit altered innate immune responses, including attenuated macrophage activity and impaired neutrophil recruitment, which subsequently alters the intestinal integrity, allowing microbial agents to pass through the mucosa(37). These patients also present with a population of macrophages that expresses dendritic cell markers such as CD14 and produce increased levels of pro-inflammatory cytokines, such as TNF-α and IL-6(60). In sharp contrast, dendritic cells monitors the environment and relay signals to initiate appropriate adaptive responses(61). Dendritic cells contact in proximity with the microbiome, in part by a CX3CR1 (CX3-C motif chemokine receptor 1) dependent mechanism. The genetic deletion of CX3XR1 results in decreased numbers of lamina propria macrophages and increased translocation of luminal bacterial to mesenteric lymph nodes(61). In IBD patients, dendritic cells have been found to accumulate in the mucosa(62). The blockage of CD40/CD4L interactions between dendritic and effector T-cells, prevents T-cell mediated experimental colitis(62).

Leukocytes migrate to the inflamed gut through the binding of integrin molecules located on the surface of leukocytes to cellular adhesion molecules expressed on the surface of IECs. IECs respond to inflammatory signals producing chemokines to attract leukocytes to inflammatory sites(63). The integrins a_Lb_2, a_4b_1, a_4b_7 and a_Eb_7 are heterodimeric receptors expressed on the surface of circulating leukocytes, capable of interacting with different adhesion molecules in the intestine(63). These molecules constitute pharmacological targets, and prevent leukocyte migration to the intestine and control inflammation in IBD patients(63). New pharmacological therapies which target endothelial cell cellular adhesion molecules and prevent lymphocyte egression from lymph nodes are being developed(64).

The common feature of CD and UC pathology is the dysregulated T-cell immune responses(37). The intercalated transmural inflammation of CD results from an excessive Th1 and Th17 cell response, while the uniform mucosal inflammation observed in UC is secondary to a Th2 cell type-like cytokine profile. In UC, increased secretion of IL-5 (Th2 cell-specific cytokine) is associated with more efficient activation of B cells and induction of immune response, compared with the Th1 cell response observed in CD(37). Interestingly, UC is associated with the presence of CD1d-restricted non-classic natural killer T cells that produce IL-13, which are found in increased numbers in the lamina of UC patients but not in those with CD. In CD, the differentiation of Th1 and Th17 cell types occurs in response to the production of IL-12, IL-23, IL-18 and transforming growth factor -b by macrophages and antigen presenting cells (APCs)(37). In response, these T cells produce and secrete pro-inflammatory cytokines such as interferon γ (IFN-γ), TNF-α, IL-17 that stimulate APCs, macrophages, fibroblasts and endothelial cells to further produce pro-inflammatory cytokines(37). Studies in mice have shown that genetic deletion of the IL-12 p40 subunit and IL-23 induces resistance to experimentally induced colitis, suggesting that these cytokines are core features of the CD inflammatory pathway(65). There are currently many clinical trials targeting the IL-23 specific p19 subunit and the shared p40 subunit of IL-12 and IL-23 in CD patients(66). Interestingly, drugs developed to block IL-17A worsen the inflammation in patients with CD, because Th17 cells are implicated in important homeostatic functions in the intestine, such as, the protection of barrier integrity and the regulation of Treg function which control immune activation and decrease inflammatory responses(66). Tregs and Th17 exert opposing activities but arise from a common precursor upon transforming growth factor b (TGF-b) stimulation. TGF-b signaling contributes to normal homeostasis by promoting Treg differentiation in naïve lamina propria CD4 T-cells(67).

In IBD, in response to TGF-b signaling and other signals, Th17- cell differentiation is promoted. Thus, the inflamed gut uses TGF-b pathways to promote T cells to induce both pro-and anti-inflammatory actions, depending on the presence of cytokines and microbial products that may consequently affect initiation, persistence, and relapses in human IBD(67). It has been shown that many genes required for Treg and Th17- cell differentiation are implicated in the pathogenesis of IBD(68). In experimental models, inflammation driven by Th17 cells leads to colitis. Loss of function mutations of the key transcription factor FOXP3 (forkhead box P3) induces IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is accompanied by intestinal inflammation. Tregs

are considered crucial mediators of modulating the pathology of UC and has been considered as potential therapeutic targets to control excessive inflammatory responses in IBD(69).

Immunotherapies are currently developed to target downstream signaling pathways associated with different cytokines. For example, Janus kinases (JAKs) are a family of tyrosine kinases (JAK1, JAK2, JAK3 and TYK2), that mediate intracellular communication between cytokine receptors and nuclear signals. These molecules are the main mediators between cytokine receptors and intranuclear proteins, such as, signal transducers and activators of transcriptions factors (STATs). These transcription factors are combined in pairs with different JAKs in order to mediate the intracellular effects of specific cytokine pathways, and therefore constitutes potential therapeutic targets in the modulation of intestinal inflammation(70). In the case of IL-12 and IL-23, which are important mediators in the pathogenesis of CD, JAK2 and TYK are activated by membrane receptors, which subsequently recruit STAT3 and STAT4 to deliver signals in the nucleus, associated with the control of Th1/Th17 cell responses. Recently, it has been reported that JAK inhibitors have promising results in controlling inflammation in refractory UC(70).

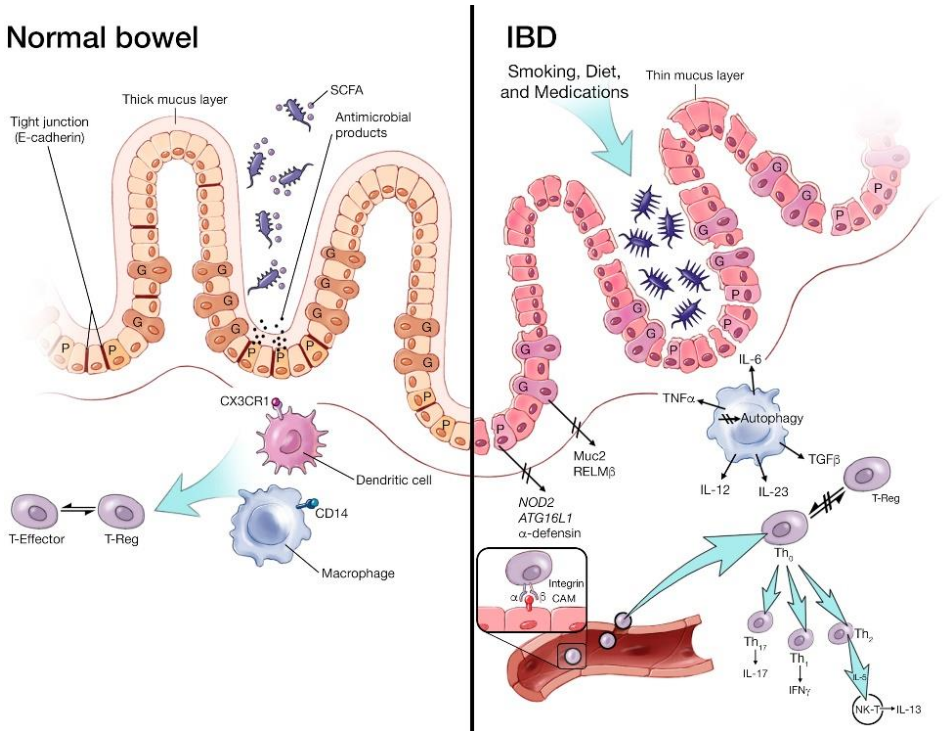

Figure 4. Mechanisms implicated in the pathogenesis of IBD. *Environmental factors such as smoking, diet and medications, can induce microbial dysbiosis with a decrease in the number of bacteria producing SCFA and an increase in Proteobacteria. In IBD, the mechanisms that maintain intestinal barrier integrity are disrupted, with down-regulation of epithelial cadherin (E-cadherin) in tight junctions and development of a thin mucus layer. Goblet and Paneth cells function abnormal, with the latter to present disrupted secretion of antimicrobial products and mutations in NOD2 and ATG16L1 genes. Innate immune mechanisms are disrupted, with colonic macrophages expressing CD14 and dendritic cells are presented with defective CX3CR1 antigen presentation and impaired autophagy. In IBD, the balance between effector and regulatory T cells (T_{reg}) appears disrupted, resulting in uncontrolled activation of different T-cell lineages that migrate into the inflamed intestine. G: goblet cells; IFN-γ: interferon γ;NK-T: natural killer T cells; P=Paneth cells; Th: T-helper cells; TGFβ:tumor growth factor β; TNFα: tumor necrosis factor α. This figure was adapted from Ramos et al. Mayo Clin Proc. January 2019;94(1):155-165*

1.4. The Co-morbidity between psychiatric disorders and Inflammatory Bowel Disease (IBD).

IBD patients commonly experience psychopathologies such as depression and anxiety(4). The prevalence of classical psychiatric disorders is significantly higher in IBD patients than in healthy people(71). A growing body of evidence suggest that the psychosomatic symptoms present in IBD patients may be a consequence of alterations in biological processes, involving immune-inflammatory, oxidative and nitrosative stress pathways, dysregulation of the tryptophan pathway, deficits in HPA axis activation/de-activation and disruption of the composition of the commensal gut microbiota(72).

There are numerous epidemiological studies showing that individuals with IBD exhibit lower quality of life (QOL), diminished psychological functioning and well-being than the general population(6). The development of anxiety and depression is more prominent in IBD patients with increased disease activity scores than those in remission(73). In a recent study, *Lix et al.* have shown that IBD patients with decreased disease activity, experience less pain and anxiety(74). Of course, it should be stated that in the same cohort of IBD patients the variability in the psychological outcome was profound(74). The same authors found that patients with penetrating or structuring disease had significantly lower scores on the Inflammatory Bowel Disease Questionnaire and the SF-36 physical health component than patients with the active disease(74). These data suggest that disease activity rather than the disease itself plays a pivotal role in the physiological outcome.

In a large-scale population study in Canada, a cohort of patients with IBD was compared across a number of psychological and QQL domains with a cohort of non-IBD patients originating from the same community(75). This study found that psychological parameters had greater contribution to health perception for the IBD patients compared with the non-IBD patients(75). Interestingly, the cohort of IBD patients with inactive disease were quite similar in the non-IBD sample and exhibited higher mastery level(75). Furthermore, the distress levels were significantly higher for those with IBD (both UC and CD) than without IBD. The sample with the inactive disease did not exhibit any more distress than the control sample. Also, less than 10% of those with inactive disease saw themselves as being in poor health, similar to the control (non-IBD) sample(75). These data indicate that patients in remission have psychological benefits and improvement in their physical symptoms. An interesting finding raised from the IBD cohort where they reported modestly higher levels of mastery in contrast with the control (non-IBD) cohort. The study subjects had lived with IBD for an average of 4 years, so it is assumed that chronic disease strengthened their sense of mastery as they confronted the illness. The longitudinal tracking of mastery of these patients was associated with modest increases over time, with levels highest for those with inactive disease. Therefore, although living with chronic disease may have negative effect psychologically, psychological variables may be strengthened(76). IBD patients with chronic disease commonly experience higher lifetime rates of panic, generalized anxiety, and obsessive-compulsive disorders as well as major depression(76).

Another important aspect of the psychological outcome in IBD patients revealed in a large-scale epidemiological study of health records among residents of southern England with UC

and CD(77). This study showed that during the first year after the diagnosis of IBD the rates of treatment for anxiety and depression was very high among the IBD patients. Moreover, in a sample of patients with IBD was found that disease severity and psychological symptoms contributed independently to impaired QOL(4,77). Psychological morbidities are believed to exacerbate the disease severity through a number of mechanisms, including decreased adherence to treatment recommendations, suppressed immune system functioning, altered ANS (eg. reduced vagal tone with excessive sympathetic activity) or HPA hyperactivity(78). Specifically, depression can negatively affect the course of the disease activity. Recently, a prospective study of patients with IBD found that those with clinically significant depressive symptoms at baseline had relapses that occurred sooner (median time to first relapse was 97 days vs 362 days in those without depression) and more frequently during the following 18 months(78). Another study with CD patients found that major depression was a risk factor for failure to achieve remission with an anti-inflammatory treatment. The presence of depression and anxiety failed to improve the therapeutic outcome of IBD. On the contrary, better psychological adjustment was associated with greater bowel and systemic health, less pain, less perceived stress, symptoms tolerance and increased engagement in activities(78). An important question that arises from the above studies is if the existence of a mood disorder predisposes the development of IBD. This question was assessed by *Walker et al*. where they found that the lifetime prevalence of major depression in patients with IBD was 27.2 % versus 12.3 % higher than the healthy individuals(78). About one-half of those with a mood disorder (54 %) experienced a first episode of depression more than 2 years before the onset of IBD. *Walker et al.* compared the lifetime prevalence of psychiatric disorders in a population-based cohort of 351 patients with IBD with a population-cohort of healthy individuals taken from the same community (the Canadian Community Health Survey)(78). A growing body of evidence has shown that IBD patients are benefited by treatments with conventional anti-depressant drugs(6). Antidepressants in patients with IBD seem to reduce relapse rates, use of corticosteroids and endoscopies in the year after their introduction(79).

1.5 Animal models of Inflammatory Bowel Disease (IBD).

Experimental models of IBD has been developed to study the molecular mechanisms that mediate the development and progression of both UC and CD(80). Different animal models have been developed containing transgenic or gene targeted (conditional or knock out) mouse strains (eg IL-10-/- mouse strain) which display some features of the human IBD(80). Mice deficient in the cytokine IL-10 or its receptor develop spontaneous colitis resembling the severe transmural and discontinuous inflammation in CD(81). Thus, loss-of-function mutations in the IL-10 or the IL-10 receptor gene are useful models to study the role of this signal transduction pathway in the pathogenesis of the early-onset IBD. It is known that CD affects mainly the lower part of the small intestine(81). For this reason, a strain has been developed to study the development of ileitis in which the pro-inflammatory cytokine TNF-α is overexpressed (TNFΔARE mice)(82).

However, most studies commonly use chemically induced models of colitis (83). These models exhibit some advantages, such as, the ability to perform experiments in immunocompetent mice, in contrast with other murine models which require cell transfer

(T-cells) in immunodeficient host animals. Also, using chemically induced models of colitis, one can avoid the disadvantages of genetically engineered colitis models, such as, the presence of developmental abnormalities due to the genetic defect or the high inter-individual variability in the penetrance and activity of gut inflammation.

A plethora of studies suggest that chemically induced mouse models constitute useful tools to mimic some immunological and histopathological aspects of human IBD(83). Despite the fact that these models cannot recapitulate the whole complexity of human IBD progression and pathology, they are considered valuable *in vivo* tools to understand the various aspects of the disease pathology. Furthermore, the use of chemically induced models of colitis offer highly applicable models for the development of novel pharmacologically treatments. The most common chemically induced models of colitis are the dextran sodium sulfate (DSS), the trinitrobenzenesulphonic acid (TNBS) and the oxazolone model(83). Most studies concerning the effects of gut inflammation on brain and behaviour, commonly use the DSS and TNBS models of colitis.

1.5.1 DSS colitis.

Administration of DSS via the drinking water induces severe colitis characterized by excessive weight loss, diarrhea, ulcer formation, loss of epithelial cells and infiltrations with mononuclear cells, resembling some characteristic features of the human UC(84). It has been shown that DSS exert its actions through a mechanism where it associates with colonic-medium-chain-length fatty acids, abundantly present in the chow which are absorbed and metabolized by epithelial cells. DSS causes epithelial injury and compromise epithelial barrier integrity(85). The latter, induce a rapid inflammatory immune response through the expose of the epithelium in mucosal and microbial components. This model is appropriate to study the effects of acute and chronic colitis on brain and behaviour. Repeated epithelial damage and wound repair phases induced by the administration of DSS for several cycles cause a non-self-limiting chronic form of colitis which recapitulates some features of the human UC(80).

The induction of acute DSS colitis is dependent on the induction of innate immune mechanisms as immunodeficient mice (SCID) develop strong colitis(83). Therefore, acute DSS colitis is used to analyze the innate immune system mechanisms in the development of intestinal inflammation and the re-establishment of barrier integrity. However, it has been shown that as the DSS colitis progresses, T cells accumulate in the inflamed mucosa where they exhibit pathogenic characteristics (86).

1.5.2 TNBS colitis.

In this model, colitis is induced by intrarectal administration of the haptenic substance TNBS together with ethanol(80). Ethanol is essential to provide access to intestinal epithelial cells thus impairing barrier function and allowing TNBS to penetrate the bowel wall. The mechanism of action of TNBS is mediated through the haptenization of colonic or microbiota-derived proteins with subsequently generation of TNP-specific $CD4^+$ cells and antibodies(86). TNBS model of colitis is suitable for studying the adaptive immune system, as T cells plays a key role in the pathogenesis of TNBS colitis. The intensity and the length of the inflammatory process are dependent on several factors such as the genetic background

of mice and the presence or absence of T-cell-activating bacterial strains. Depending on the microflora and the strains used, different cytokine profiles have been described, including activation of Th1, Th2 and Th17 cytokines(83,86). In the histological level, TNBS exert features of transmural inflammation with infiltrates of macrophages, neutrophils and lymphocytes as well as features of colonic patch hypertrophy(80). As these immunological and histological features recapitulate aspects of the human CD, TNBS colitis has been considered as a model to study a variety of aspects potentially relevant to this disease.

1.5.3 Oxazolone colitis.

Oxazolone colitis is induced by intrarectal administration of a hapten reagent(87,88). The acute feature of inflammation is characterized by ulcerations and lamina propria infiltrations of neutrophils, macrophages and lymphocytes. The histological features of oxazolone colitis are characterized by epithelial damage and infiltration of neutrophils, macrophages and lymphocytes in mucosa and submucosa as well as preferential inflammation of the distal colon and an elevated production of type 2 and type 9 cytokines (IL-4, IL-5, IL-9 and Il-13) by lamina propria T cells(87,88). Also, natural killer T cells which secret IL-13 play key role in the disease development. It has been shown that IL-4R signaling to T cells and B cells and IL-9 production by T cells have been demonstrated to contribute to the disease. This model is suitable for studying the type-2- and type-9-related immune responses during intestinal inflammation(87,88).

1.5.4 DSS colitis and CNS alterations

A plethora of studies have extensively used the chemical induced models of colitis to study the functional link between psychiatric disorders and gastrointestinal pathologies. In this part of the introduction, we focus on some studies used the DSS induced model of colitis to explore the effects of intestinal inflammation on behaviour as well as on molecular mechanisms that it is speculated to mediate the effects of intestinal inflammation on the brain.

1.5.4.1 Anxiety/Depression-like alterations.

Acute DSS colitis has been shown to increase anxiety and depressive-like behaviours in rats. In these studies, *Chen et al.* found that rats with acute DSS colitis spent less time in the open arms of the elevated plus maze (EPM) and presented with increased immobility time in the forced swimming test (FST), indicating anxiety and learned helplessness respectively(89). Also, rats with DSS colitis had decreased preference in saccharin in the sucrose preference test and reduced social interaction, suggesting anhedonic behaviours and social avoidance, characteristics of depressive-like behaviours(89). These behavioural effects were reversed by prolonged desensitisation of the transient receptor potential vanilloid 1 (TRPV1), expressed in colonic afferent neurons by infusion of capsaicin and resiniferatoxin (TRPV1 stimulator)(89).

More interestingly, the behavioural effects of DSS colitis seems to be sex dependent. In a recent study, *Painsipp et al.* used male and female mice to evaluate the effects of intestinal inflammation on behaviour at days 8, 9 and 11 post DSS colitis(90). Male mice spent less time in the open arms of the EPM, indicative of anxiety like behaviour, whereas female mice had increased immobility rates in the FST, indicative of depressive like behaviour(90). In

another study, *Emge et al.* found that during the active phase of inflammation, mice exhibited anxiety-like behaviour in the light/dark box, accompanied with impaired recognition memory as assessed by the novel object recognition test. Interestingly, these behavioural deficits were normalized 14 days post DSS (remission phase)(91). However, a more recent study has shown that following resolution of the acute colitis response, rats developed sustained anhedonia in the saccharin preference test, increased immobility in the forced swim test, reduced burying behaviour in the marble burying test, and mild signs of anxiety in the elevated plus maze and light/dark box(92).

1.5.4.2 Effects of experimental colitis in BBB permeability

Many studies have shown that systemic inflammation can disrupt the BBB integrity, allowing the influx of pro-inflammatory cytokines and peripheral immune cells into the brain parenchyma(22). Experimental colitis has shown to increase the permeability of BBB in low molecular weights (such as fluorescein, 376 kDa) but not in higher ones like IgG (MW 156, 000 kDa)(93). In these studies, the authors identified the circumventricular organs (e.g the organum vasculosum of the lamina terminalis, subfornical organ and median eminence) to be the sites of high permeability(93). In another study, *Sans et al.* using multiple models of experimental colitis such as the DSS and TNBS model, IL-$10^{-/-}$ mice and mice reconstituted with CD45RB high T-cell, have found a significant increase in the expression levels of the vascular cell adhesion molecule-1 (VCAM-1) in the brain of all experimental models of colitis(94). They reported that mice with TNBS colitis had increased expression of the intercellular adhesion molecule-1 (ICAM-1) in their brain compared to mice with DSS colitis(94). The changes in the expression of the adhesion molecules were not accompanied by infiltration of leukocytes. These experimental findings indicate that BBB disruption during intestinal inflammation affects the brain, leaving it vulnerable in inflammatory stimuli coming from the periphery.

1.5.4.3 The cytokine profile of pro-inflammatory cytokines in the CNS during DSS colitis

Cytokines are implicated in the pathogenesis and progression of IBD(37). Circulating cytokines reach the brain through the BBB and the circumventricular organs and activate the local glia cells and neurons to produce pro-inflammatory cytokines which exert neurotoxic action with detrimental effects on brain function(95). One common therapeutic strategy in IBD is the use of antagonists for the TNF-α cytokine(37). This highlights the key role of cytokines in the pathology of IBD as well as in depression and other psychiatric illnesses.

The transcriptional levels of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β and iNOS were upregulated in the substantia nigra of rats with 7 days of DSS colitis(96). Acute DSS colitis in combination with water avoidance stress (WAS) induces the levels of IL-1β, IL-6, IL-17A, IL-18, and TNF-α and the growth oncogene factor GRO-α in the plasma, hypothalamus, amygdala and hippocampus(97). DSS colitis alone, induced the levels of plasma cytokines while no differences were observed in the levels of cytokines in the brain regions of hypothalamus, amygdala and hippocampus(97). These data suggest that WAS is required to cause a brain inflammatory response in mice with DSS colitis. Prolonged

immobility in mice with DSS colitis during WAS were associated with brain region-dependent alterations in the expression genes associated with energy homoeostasis [neuropeptide-Y (NPY) and the NPY receptor Y1], stress pathway activation (CRH and CRH 1 receptor and the glucocorticoid receptor) and neurogenesis (BDNF)(97). The authors proposed that alterations in gut–brain signaling may be responsible for the observed behavioural changes in response to stress in DSS animals. Recently, a study performed by *Zonis et al.* found an increase in circulating IL-6 in mice with acute DSS colitis, accompanied with a parallel increase in hippocampal microglia and astrocytes(98). Also, in mice with chronic DSS colitis the transcriptional levels of TNF-α and IL-1β were upregulated which was accompanied with increased protein levels of GFAP and IL-6 in the hippocampus(98).

1.5.4.4 DSS colitis and HPA axis activation

We previously discussed that gastrointestinal disorders regulate the activation of HPA axis. There are very few data available on the HPA axis activity in IBD patients(6). Some studies suggest that IBD patients in response to a psychosocial stressor exhibit hyporeactive HPA axis(6). There are data suggesting an uncoupling between the HPA axis and the sympathetic nervous system (SNS) in patients with IBD. There are cases that serum levels of cortisol did not correlate with plasma neuropeptide Y, a marker of the SNS(91). Such an uncoupling has also been observed in patients with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus(6).

Recently, it has been shown that acute DSS colitis increased basal and post-stress (90 min) levels of circulating corticosterone – an index for increased HPA axis activity(97). Other studies suggest that DSS administration alone do not increase the plasma levels of corticosterone. However, the combination of WAS and DSS colitis causes a profound upregulation of plasma corticosterone levels(97).

Recently, it has been shown that stressors prior to DSS colitis, aggravates the severity of induced colitis(99). Chronic psychosocial stress induced by repeated exposure to social defeat stress (SD, 2 hours) and overcrowding (OC, 24 hours) for 19 consecutive days aggravated the DSS- induced colitis(99). This was reflected by the increased body weight loss, the elevated cytokines in the mesenteric lymph node cells and the decreased colon length(100). Moreover, the group of stressed mice had lower survival rates during the remission phase of colitis(100). These findings suggest that exposure to psychosocial stressors prior to DSS colitis, results in adrenal insufficiency and increases the severity of acute intestinal inflammation which subsequently affects the repairing mechanisms. HPA axis is an important regulator of the activation/de-activation of the immune system and its dysregulation could cause pathological impairments with debilitating effects on cognitive function and mood(71).

1.6 Adult neurogenesis in the mammalian brain

Adult neurogenesis is a dynamic process where new neurons are continuously generated in specific regions of the adult mammalian brain and functionally integrate into the existing neural circuits(101). Adult neural stem cells (NSCs) reside in two major niches in the adult mammalian brain: the subventricular zone (SVZ) lining in the lateral ventricles and the

subgranular zone (SGZ) within the dentate gurus (DG) of the hippocampus(101). Adult NSCs in the SVZ (B cells) extend a basal process which terminate on blood vessels whereas their apical processes pokes through the ependymal cell layer to contact the cerebrospinal fluid (CSF) in the ventricle(102). Type B NSCs give rise to transient amplifying progenitors termed C cells, which divide a few times before becoming neuroblasts (A cells)(103). Neuroblasts then migrate into the olfactory bulb through the rostral migratory stream and differentiate into different subtypes of interneurons. In the SGZ of the DG, adult NSCs termed Radial glia-like NSCs (RGLs or type 1cells) give rise to intermediate progenitor cells (IPCs)(104), which exhibit limited rounds of proliferation before generating neuroblasts(105). Then, neuroblasts migrate tangentially along the SGZ and develop into immature neurons, which migrate radially into the granule cell layer (GCL) to differentiate into dentate granule neurons(105). Adult neurogenesis is regulated by intrinsic and extrinsic factors at all levels, including the proliferation of adult NSCs or progenitors, differentiation and determination of progenitor cells and the survival, maturation, migration and integration of new neurons in the functional neuronal circuitries(101). Moreover, these cells may be required for certain forms of brain function involving the olfactory bulb and the hippocampus.

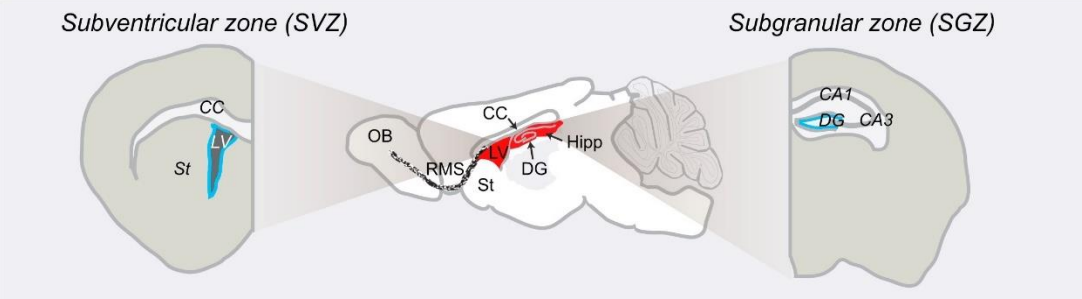

Figure 5. Neurogenesis in the adult brain. *Sagittal view of the adult rodent brain. There are two major niches where adult NSCs reside: the subventricular zone (SVZ) and the subgranular zone (SGZ). The SVZ is located along the lateral ventricle in the forebrain (left), while the SGZ is located in the hippocampus (right) along the dentate granule cell layer where it abuts the hilus. CC, corpus callosum; DG, dentate gyrus; Hipp, hippocampus; LV, lateral ventricle; NSC, neural stem cell; OB, olfactory bulb; RMS, rostral migratory stream; SC, stem cell; St, striatum. This figure was adapted from Bond and Song et al. Cell Stem Cell, 2015.*

1.6.1 Adult neurogenesis in the human brain.

The pioneering work of Altman and Das was the first to suggest the existence of neurogenesis in the adult mammalian brain(106). Until then, neuroscientists believed that the birth of new neurons in the mammalian brain was restricted to embryonic and early postnatal development. It was assumed that newborn cells in the brain would destabilize the existing information and disrupt the pre-existing neural circuits. The initial breakthrough in

the field of neurogenesis came with the use of thymidine analogues such as bromodeoxyuridine (BrdU), to label dividing cells and their progenitors. BrdU and other halogenated thymidine analogues, such as IdU or CldU, are incorporated into the DNA of dividing precursor cells and are detected immunohistochemically(101). The combined use of antibodies detecting BrdU-labeled nuclei with neuronal markers such as NeuN indicate that the newborn neuron has originated from a cell that underwent division at exactly the time at which BrdU was applied, since BrdU has a short biological half-life(105). In 1998, *Eriksson et al.* applied this methodology to detect proliferating neural stems in the brain of patients with late stage tumors who did not receive any kind of pharmacological treatment(107). Post-term analysis in five brains of these patients revealed proliferating ($BrdU^+$ cells) neural stem cells in the human dentate gyrus(107). Recently, *Frisen and colleagues* applied a technique whereby rates of newborn neurons could be estimated by taking advantage of increased radioactive carbon (^{14}C) in the atmosphere due to above – ground nuclear testing (108). The radioactive carbon incorporates into DNA in a similar way as the thymidine analogues. This technique enables estimates of overall levels of neurogenesis, showing a turnover of granule cells at 1.75 % a year(108). This strategy did not confirm the existence of ongoing neurogenesis in the human olfactory bulb (OB). However, it appears that human NSCs in the SVZ subregion give rise to a subset of striatal interneurons, a neurogenic route that is absent in rodents(108).

Other key supportive evidence for the existence of adult neurogenesis in the human brain came from the study of *Palmer et al.* where they managed to culture stem cells with neurogenic potentials from the adult human hippocampus(109). In addition, several studies have used immunocytochemistry to detect cells expressing cell proliferation markers in human postmortem brains(110–113). For example, *Knoth et al.* using a combination of 14 neurogenesis markers (eg DCX and PSA-NCAM) reported neurogenesis in 54 human samples across the lifespan of 0 to 100 years (114). In the same conclusion came the recent study conducted by *Boldrini et.al* (114) using the same approach as *Knoth et al*. Interestingly, *Boldrini et al* by employing additional validation methods did not find an association between labelled cells and increasing age(114). In contrast to previous studies, they applied stereology, a method for unbiased quantification within a tissue volume. It is of great interest to notice here that at the same period of these findings, the group of *Sorrels et al.* reported that neurogenesis in the human hippocampal dentate gyrus drops to undetectable amounts during childhood, and that the human hippocampus function differently from that in other species, in which adult neurogenesis is conserved(115). These discrepancies were attributed to the limitations of marker studies, of quantitative techniques used as well as of the species differences in neurogenesis process(116). Despite these discrepancies, it is clear that additional methods should be used to study the generation of new neurons in adult humans. Methodological tools like single-cell RNA sequencing, as well as non-invasive imaging strategies such as magnetic resonance imaging (MRI) and positron emission tomography (PET), is likely to provide valuable information(116).

1.6.2 Adult hippocampal neurogenesis

The hippocampus constitutes an important region for mood and cognition. The unique feature of this brain region is the existence of neurogenesis(105). Adult hippocampal

neurogenesis generates only one type of neuron: granule cells in the dentate gyrus(105). Granule cells are the excitatory principal neurons of the dentate gyrus. They receive input from the entorhinal cortex and send their axonal projection along the mossy fiber tract to area CA3 of the hippocampus, where they terminate in large synapse-and interneuron-rich structures called boutons(105). These axonal projections provide excitatory input to the pyramidal cells residing in the CA3 region(105). Granule cells neurons fire very sparsely and their activity is modulated by interneurons in the DG and the hilus area of the DG. In the DG, adult neural stem cells and progenitors reside in a narrow band of tissue between the hilus and the granule cell layer which is called subgranular zone (SGZ)(117). This layer of tissue - which is called niche - contains the microenvironment that is permissive for neural stem cells to develop(117). The niche is comprised by precursor cells, their intermediate progeny, immature neurons, newborn neurons, glial cells, endothelial cells, immune cells like microglia and macrophages as well as by extracellular matrix. As this niche is highly vascularized it has also been called as the "vascular niche"(118).

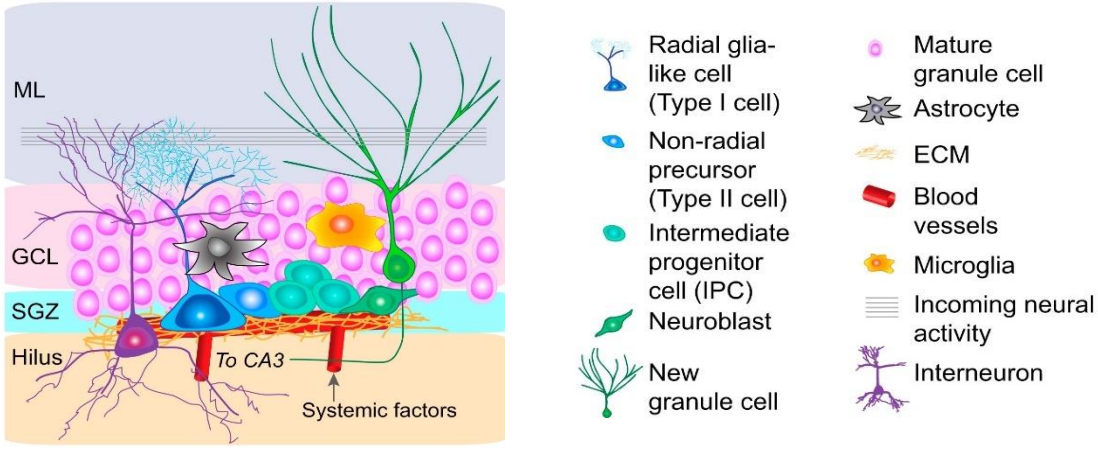

Figure 6. The neurogenic niche of the hippocampus. *This schematic diagram depicts the cellular and molecular components of the SGZ niche. Specifically, the neurogenic niche of hippocampus is consisted by Radial glia-like NSCs (type 1 cells) that extend a radial process through the granule cell layer (GCL) into the molecular layer (ML) of the DG. Radial glia-like NSCs generate intermediate progenitor cells (IPCs), which generate neuroblasts, with these progenitors to be closely associated with the vasculature. Neuroblasts differentiate into dentate granule cells and migrate along the GCL of the DG. Other components of the niche are astrocytes, microglia and interneurons that contribute to the cellular architecture of the niche. ECM, extracellular matrix; EZ, ependymal zone; GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone. This figure was adapted from Bond and Song et al. Cell Stem Cell, 2015.*

1.6.2.1 Developmental phases of adult hippocampal NSCs

Adult hippocampal neurogenesis can be divided into four developmental phases: 1) a precursor cell phase, 2) an early survival phase, 3) a postmitotic maturation phase and 4) a late survival phase.

1.6.2.2 Precursor phase.

The precursor phase refers to the expansion of the pool of cells that might differentiate into neurons(105). These precursor cells exhibit morphological and antigenic characteristics of radial glia cells(104). The cell body of these cells is found in the SGZ and their process

extends into the molecular layer of the DG and are considered quiescent (G_0 phase of the cell cycle)(104). However, the population of these radial glia like cells (RGLs) is relatively heterogeneous. RGLs which are referred as type 1 cells and express GFAP, Nestin and Sox2 exhibit NSCs properties (self-renewal and differentiation)(119). Clonal analysis of individual RGLs has revealed self-renewal and multipotent capacities(119). Also, it has been proposed that these RGLs cells exhibit alternative properties(120,121), suggesting the existence of heterogeneity among RGLs(122). More interestingly, non-radial precursors expressing Sox2 have also been proposed to exhibit multipotent characteristics, and other proliferating cell populations may act as NSCs depending on certain conditions(122). Single-cell gene expression analysis has shown that only a few genes were specific to quiescent NSCs(123). These results point to a more complex scenario for the developmental sequence in the adult hippocampal lineage than previously simplified models might have suggested. Furthermore, two recent studies demonstrated that single NSCs are not long-term self-renewing(124,125), supporting the emerging concept that NSCs persist only at a population level. The origin of adult NSCs is to a large extent unknown. One proposed model suggests that adult NSCs originate from the whole length of the dentate neuroepithelium, which produces both embryonically generated granule neurons and adult NSCs. Another model proposes that adult NSCs may originate during late gestation from a population of sonic hedgehog (Shh)-responsive cells residing in the ventral hippocampus. The descendants of these cells then relocate into the dorsal hippocampus to become the source of adult NSCs in the SGZ(126).

RGLs type 1 cells upon activation give rise to intermediate progenitor cells, referred as type 2 cells(105). These cells are highly proliferative(105). Also, a proportion of type 2 cells expresses glial markers but lack the characteristic morphology of the typical RGLs type 1 cells(105). Type 2 cells can be divided further in two distinct subtypes: type 2a and type 2b. Type 2a cells express the intermediate filament Nestin and the transcription factor Sox2(105). Type 2b cells express mainly the transcription factors Prox1 and NeuroD1(127). Prox1 is considered specific to granule cell development and knock out of this transcription factor abolish adult neurogenesis at this stage(128). Also, type 2b cells express doublecortin (DCX)(105). DCX is expressed at the proliferative stage, even after Nestin has been downregulated. Normally, DCX- expressing cells which are referred as type 3 cells show little proliferative activity(105). Under pathological conditions, such as experimental seizures, they show a profound increase in their proliferation capacity(129). Furthermore, DCX overlap with PSA-NCAM expression - a widely used marker for adult neurogenesis. DCX expression, extends from a proliferative stage, through a cell cycle exit stage to a stage of postmitotic maturation that lasts 2 to 3 weeks (130–132).

Also, type 2 cells are characterized by their expression of Tbr2, a transcription factor that during embryonic cortical development identifies the basal progenitor cells, which maintain self-renewing properties and can differentiate into neurons(133). Tbr2 suppress the expression of Sox2 and is critical for the transition from stem cells to intermediate progenitors(134). At type 2 cell level, the first fate choice is made and it must occur on the level of type 2a cells(105). This is clearly illustrated by the fact that later types of neural stem cells express NeuroD1 and Prox1 and there is no overlap between astrocytic markers

and NeuroD1 and Prox1 at any time point beyond type 2a cells. Moreover, type 2 cells receive the first synaptic input, which is GABAergic(135,136). Type 2 cells respond rapidly to physiological stimuli such as voluntary wheel running or pharmacological stimulants which act through serotonin-dependent mechanisms(137).

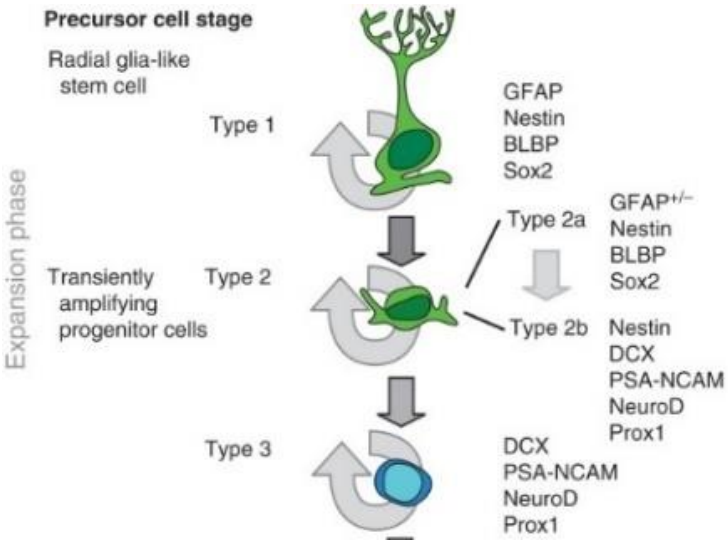

Figure 7. The precursor and early survival phase of the hippocampal NSCs lineage. *The precursor cell stage includes the activation of Radial glia-like stem cells (Type 1) that subsequently give rise to transiently amplifying progenitor cells (Type 2a/2b) and neuroblasts (Type 3). This schematic diagram depicts the developmental phases of NSCs of the hippocampus and the related markers. GFAP, Glial fibrillary acidic protein; BLBP, brain lipid-binding protein; DCX, doublecortin; PSA-NCAM, polysialilated neural-cell-adhesion molecule. This figure was adapted from Kempermann, Song and Cage, Cold Spring Harb Perspect Biol 2015;7: a018812*

1.6.2.3. Early survival phase

Early survival phase is characterized by cell cycle exit. Type 3 cells exit cell cycle and express postmitotic markers such as NeuN (RbFox3) as well as the transient marker calretinin (calcium binding protein)(132). The number of NeuN-positive new neurons is highest at very early time points and decreases dramatically within a few days due to apoptotic processes (138,139). Most newborn neurons are eliminated before they make functional connections in the CA3 area of the hippocampus or receive dendritic inputs from the entorhinal cortex(140). The main synaptic input of the newborn cells is GABAergic and at this phase GABA has excitatory properties (141). The excitatory properties of GABA are switched to inhibitory, when sufficient glutamatergic contact has been made and specifically when the new cells develop their own glutamatergic neurotransmitter phenotype(141). GABA action drives neuronal maturation and steers their synaptic integration(142). At this phase, new cells send their axons to target the CA3 area, where they form synapses with the interneurons(142). At the same time newborn neurons express the collapsing-response mediator protein (Crmp OR TUC-4) which is involved in axon path finding(142).

Early survival phase is considered important in the regulation of neuronal development, as it establishes the survival, migration and functional integration processes of new neurons in the circuitry(105). New neurons that have survived during the first 2 weeks, will stably and

persistently integrate into the functional circuitry of the DG(105). After this time point, only small changes in cell numbers occur.

1.6.2.4 Postmitotic maturation phase.

At this phase, depolarizing GABA is required to allow the formation of glutamatergic synapses (143). The exact timing of the maturation is clearly dependent on the activity in local circuits, further suggesting that adult neurogenesis is activity dependent at numerous stages of neural development. At 3 weeks after birth, the dendritic tree of these newborn neurons has already reached the outer molecular layer, where axons from the perirhinal cortex (PER) and entorhinal cortex (EC) establish synaptic contacts with newborn neurons(142,144). At this stage, dendritic spines are few and highly unstable, thus glutamatergic inputs are weak and do not induce action potentials. Also, at this phase, the axons of new neurons that previously connected to the cells in CA3, compete with existing synapses in the mossy fiber boutons(144). The size of the boutons, the number of spines enveloped and the active synaptic contacts are increased until the fourth week after birth(144). Thus, these newborn neurons are already connecting EC and CA3 and being part of the classical tri-synaptic circuit of the hippocampus(145,146). The functional maturation of new neurons progresses from a state of high input resistance to a state of normal membrane properties as of that of mature dentate granule cells.

1.6.2.5 Late maturation phase.

At this phase, new neurons are switching their calcium-binding protein from calretinin to calbidin(105). Four to eight weeks after they birth newborn neurons are considered fully integrated in the circuitry of the DG but with electrophysiological properties distinct of the mature dentate granule neurons. Specifically, they present with increased excitability and enhanced synaptic plasticity(142,145,147,148). At this time point, GABA act with an inhibitory manner onto newborn neurons, presumably due to the decrease in the expression of the chloride importer NKCC1 and the parallel increase in the chloride exporter KCC2 expression(142). Notably, 4 to 6 weeks-old neurons are highly excitable, as they are less influenced by feedback inhibition(148). The presence of the GluN2B (NR2B) subunit of the NMDA receptor and the T-type calcium channel in the membrane of the newborn neurons provides enhanced synaptic plasticity(142,145,148). The enhanced plasticity properties of new cells lead to the hypothesis that altered synaptic properties facilitates the encoding of temporal information into memories(149). Another hypothesis suggest that the increased plasticity facilitates the preferential integration of new neurons to achieve long-term changes in the network(149). Experimental evidence shows that silencing 4 week old newborn neurons, but not 2 or 8 week-old neurons, interferes with memory retrieval, suggesting that 4 week old born neurons facilitates storage of memories(145). The dendritic spines of these newborn neurons reaches a peak 4 weeks after birth but they are smaller than those from mature neurons, and reach axonal boutons already contacted by other spines, presumably from mature neurons(146). These spines commonly compete and displace synaptic contacts from mature neurons. This also occur in the CA3 region, where mossy fiber boutons originating from newborn neurons share thorny excrescences with boutons from mature neurons(150). It has been suggested that this competition may interfere with memory circuits and contribute to forgetting(151,152). Eight week-old newborn neurons are

considered indistinguishable from mature neurons(147,153). They do not show enhanced synaptic plasticity or increased excitability, but they still present higher structural plasticity and subtle morphological differences when compared with developmentally generated ones(147,153). This is illustrated by the fact that memory tasks such as Morris water Maze increase the complexity and number of spines of dendritic trees of 8-18 week old newborn neurons but not developmentally generated in the hippocampus of rats(154). Overall, 8 week old hippocampal newborn neurons are fully integrated and connected to the hippocampal circuitry and show similar electrophysiological properties as the mature neurons while they retain increase structural plasticity compared to developmentally generated neurons(105).

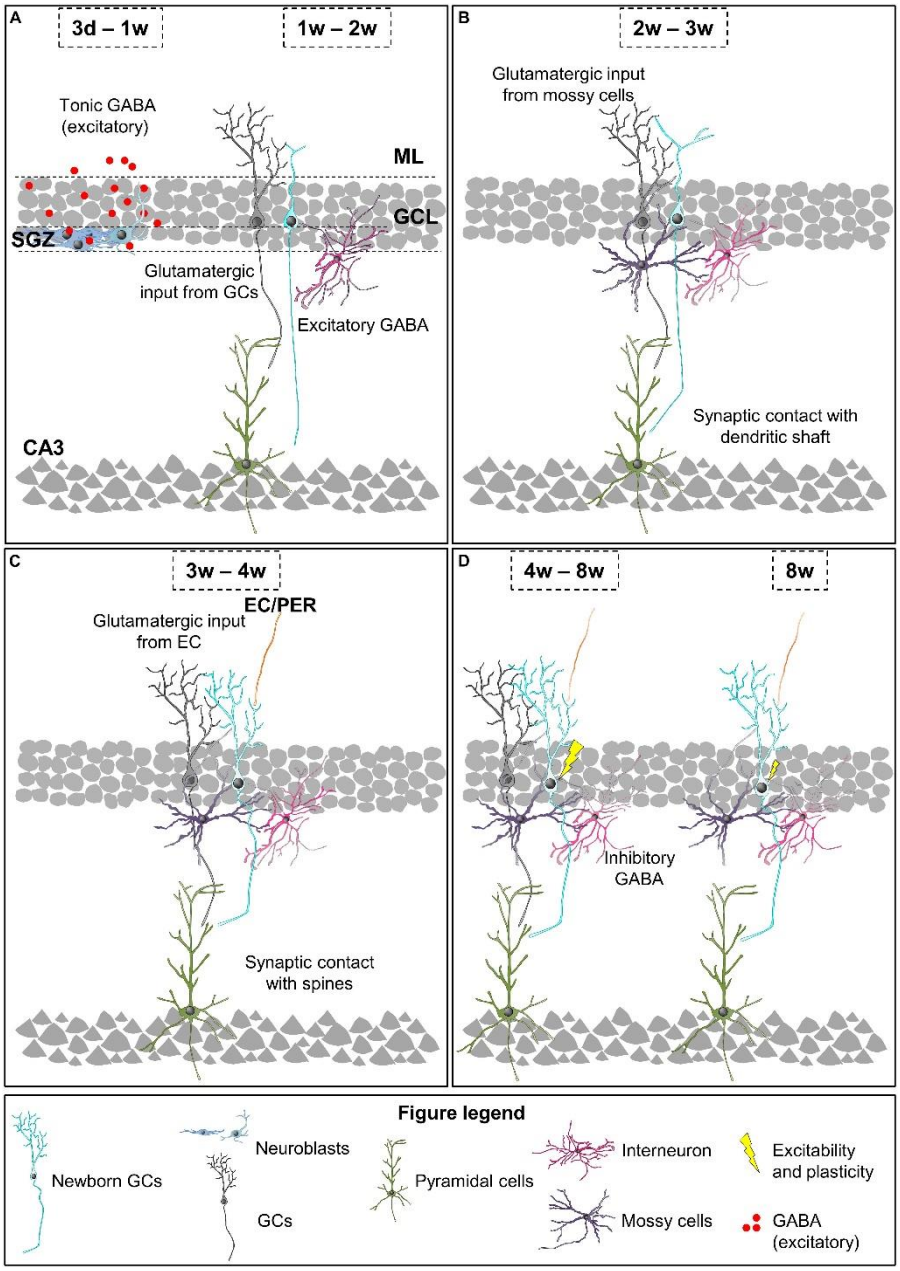

Figure 8. Maturation stages of adult newborn neurons in the circuitry of hippocampus. (A) *Neuroblasts (3 to 1 week old) express GABAergic receptors that sense GABA, which acts in an excitatory manner. Newborn neurons (1-2 weeks old) receive their first glutamatergic input from*

mature GCs and they are contacted by GABAergic interneurons, while their axons reach the CA3 region. ***(B)*** *Newborns neurons (2-3 weeks old) receive glutamatergic input from mossy cells and establish synaptic contacts with CA3 pyramidal cells.* ***(C)*** *3-4 week-old newborn neurons receive glutamatergic inputs from EC and PER, and their axons establish synaptic contacts with dendritic spines of CA3 pyramidal cells. (****D****) Newborn neurons (4-8 weeks old) are fully integrated in the hippocampal circuit and show increased excitability and enhanced synaptic plasticity. EC:entorhinal cortex; GC: granule cell; PER: perirhinal cortex. This figure was adapted from Rodriguez-Inglesias et al. 2019, Frontiers in Cell and Developmental Biology.*

1.7 The functional role of adult hippocampal neurogenesis on learning, memory and mood

The hippocampus is implicated in learning, memory and mood processes(149). In the DG of the hippocampus, the ongoing neurogenesis offers a unique plasticity property to the neural circuitry(155). Studies in rodents have shown that adult born neurons contribute to information encoding by two ways: a) by integrating information as independent units and b) by inhibiting the activity of mature dentate granule cells(149). This type of regulation is considered important for cognitive processes such as cognitive flexibility, reversal learning and pattern separation. Recent studies have shown that adult hippocampal neurogenesis is required in hippocampus-dependent tasks that involve temporal-based associations or spatial context navigation(136,156–158). On the contrary, hippocampus-independent tasks do not require hippocampal neurogenesis(159–161). The association between hippocampal neurogenesis and behaviour has been shown in studies using the immediate early genes (eg. *Fos* and *Arc*) which are markers of neuronal activity(162–165). These studies reported recruitment of 2-4 week- old adult born new neurons in the dentate gyrus circuitry during stressful conditions, spatial learning and fear memory retrieval(162). In concordance with these functional studies, electrophysiological studies suggest that new neurons may encode temporal information during their critical period(166,167). It has been proposed that the addition of new neurons in the DG constitutes an efficient method of encoding new information without disrupting previously stored information. It is now evident that adult born neurons are recruited by learning and have potential functional outcome in cognitive processes(168–170).

Apart from their function in learning processes, many recent studies have shown that adult born neurons modulate the mature dentate granule cells(171,172). More specifically, ablation of neurogenesis using X-ray irradiation increased the amplitude of spontaneous γ-frequency bursts in the dentate gyrus, as well as increased the synchronization of mature granule neurons firing. Another study using the same methodology to ablate neurogenesis, found increased levels of the intermediate early gene *Arc* in mature granule neurons during neurogenesis-dependent conflict situations(172).

On the contrary, optogenetic stimulation of young adult-born neurons resulted in the activation of local GABAergic interneurons that inhibit the mature granule cells(173). Increasing neurogenesis physically through enriched environment or physical exercise, affects the inhibition of mature dentate granule cells(172). It has been also reported that increasing hippocampal neurogenesis, reduces the number of activated mature granule

neurons during spatial exploration(172). These studies might contribute to the understanding of the role of hippocampal neurogenesis in psychopathologies, such as, anxiety and depression(149). It is well established that stress and their mediators-glucocorticoids-increase dentate gyrus synaptic currents *in vitro*(174) and *in vivo*(163). Given that, the neurogenic inhibition of the DG may be relevant for stress induced pathologies such as anxiety and depression(149).

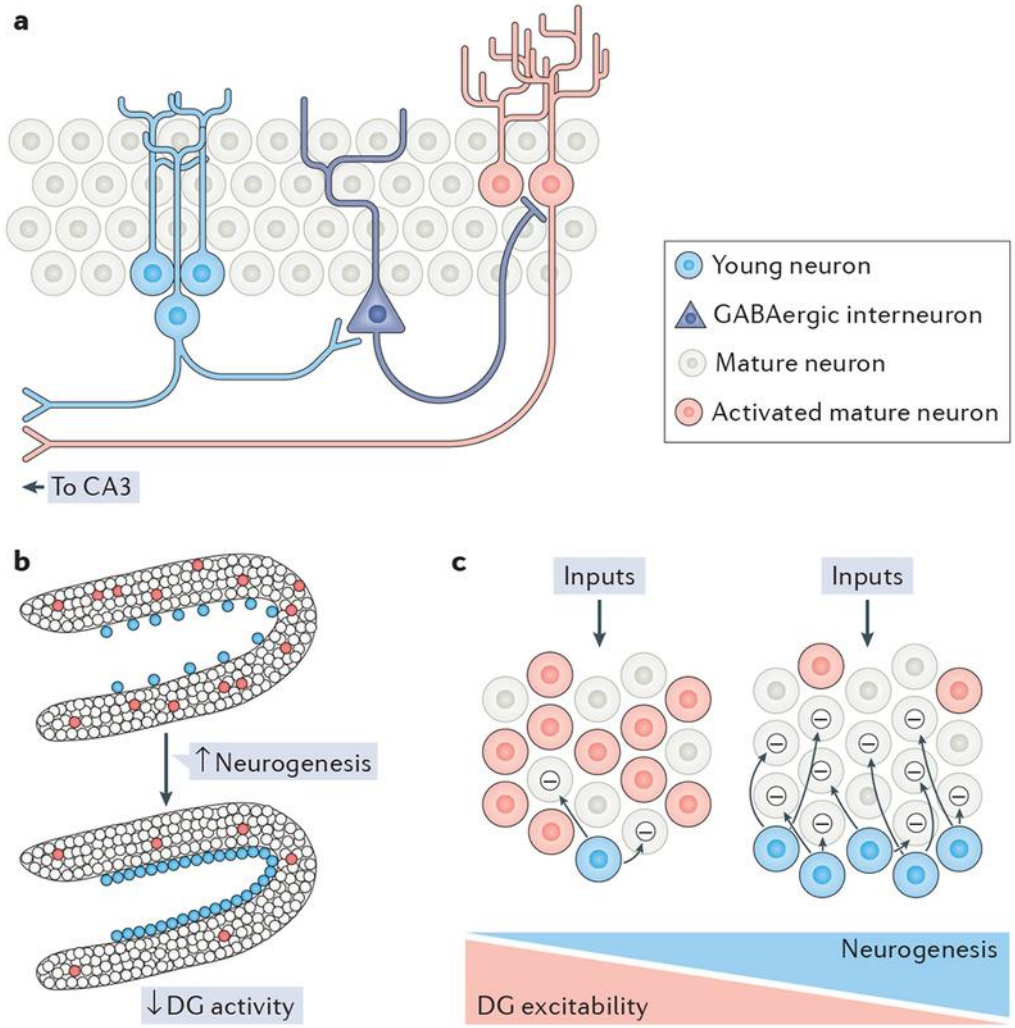

Figure 9. The dentate gyrus inhibition hypothesis. (a) *Adult newborn neurons are functionally integrated in the circuitry of the DG at 2-3 weeks of age. These neurons develop electrophysiological properties at 8 weeks of age. These neurons develop presynaptic terminals onto hilar interneurons and recruit feedback inhibition onto mature granule neurons of the DG. (b) Increased neurogenesis in mice through environmental enrichment or using transgenic strategies inhibits mature granule cells and reduces the overall excitability of the DG. Optogenetic stimulation of newborn neurons activates GABAergic interneurons in the hilus, which in turn inhibit mature granule neurons in vivo and in vitro. On the contrary, ablation of neurogenesis reduces inhibitory input to granule neurons, increases DG activity, enhances input-output coupling and expands the pool of active cells during reversal learning tasks. (c) Pattern separation relies on the sparse activation of mature granule*

cells. Newborn neurons inhibit mature granule neurons, resulting in the decrease of DG activity, during anxiogenic tasks and DG-dependent behaviours. This figure was adapted from Christoph Anacker and René Hen, Nature Neuroscience, Volume 16, 335-346.

1.7.1 Hippocampal neurogenesis and pattern separation.

It has been proposed that the function of adult hippocampal neurogenesis is related to pattern separation process(175). Pattern separation is a computational process in which highly similar contextual representations are distinguished(175). This process is mainly accomplished by the DG(175). The DG receives inputs from the entorhinal cortex which subsequently are encoded by a sparse population of granule cell neurons that form distinct representations of mnemonic information with high similarity(176,177). Then this mnemonic information is transmitted to the pyramidal neurons of the CA3 area of the hippocampus where are stored in auto-associative cellular networks that allow memory retrieval when partial cues of incomplete aspects of that memory are being detected. This process is called pattern completion(177).

One of the first pioneer studies showing the relation of adult hippocampal neurogenesis with pattern separation process was performed *by Clelland et al*. In this study, ablation of hippocampal neurogenesis, caused impairments in the spatial navigation radial arm maze task and in the spatial, but non-navigable task mouse touch screen(177). Mice with ablated neurogenesis showed impairments, when stimuli were presented with little spatial separation, but not when stimuli were more widely separated in space(177). Accordingly, increasing adult hippocampal neurogenesis enhances the ability to discriminate highly similar contexts(178–181). Inducible genetic expansion of adult neural stem cells through enhancing their survival improved performance in a pattern separation task in which two similar contexts needed to be distinguished. Specifically, increasing adult hippocampal neurogenesis did not alter the behavioural outcome on tasks such as object recognition, spatial learning, contextual fear conditioning and extinction learning but altered the efficiency of differentiating between overlapping contextual representations, indicative of pattern separation(178,181). More interestingly, discrimination of contexts that are different is not dependent on neurogenesis(149). It has been shown that adult young granule neurons is required to pattern separation process, while old born granule neurons are required for rapid pattern completion process(170,182). Despite that, mature granule cells are more efficient in the sparse encoding of contextual information due to their higher excitation threshold(170,182). In contrast, young born neurons are proposed to be ineffective at separating similar events efficiently, as their hyperexcitability make them broadly tuned to new, temporally related information(182). So, it has been proposed that adult-born neurons may function as “pattern integrators”, in order to associate similar events with each other(149). The proposed model indicates that the highly excitable young born neurons inhibit the population of mature granule cells, to achieve the sparse activation that is required for pattern separation(149).

1.7.2. Hippocampal neurogenesis and emotional memory engrams.

Engrams are memory traces that are stored in the hippocampus and constitute sufficient elements for producing fear and anxiety-like behaviors(149). For example, when mice learn to associate a context with an aversive event such as footshock, fear memory is encoded by a cellular representation in the hippocampus(183). When mice are re-exposed to the same context in which they had previously received the aversive event, the active neurons during encoding, are now recruited during the retrieval of that memory(183). On the contrary, exposure to a novel context, in which fear was never experienced, activates a different proportion of neurons in the DG(183). Ablation of hippocampal neurogenesis, either by X-ray irradiation or by optogenetically silencing adult-born granule cells, impairs engram reactivation and weakens the memory of a fearful event(151,184). Most importantly, ablation of neurogenesis impairs the reactivation of engrams only in the CA3 area of the hippocampus, suggesting that hippocampal neurogenesis regulates information processing across the hippocampal circuitry(151).

1.7.3 Hippocampal neurogenesis and cognitive flexibility.

According to *Anacker et al*. cognitive flexibility is a *"cognitive process of executive function by which previously learned behavioural strategies can be modified to adapt to changes in environmental contingencies. Enables adaptation to new situations by switching from previously held beliefs or thoughts to new response strategies"*(149). Hippocampal neurogenesis is considered to play a role in cognitive flexibility, as it allows the avoidance of interference between novel and previously formed memories(149,185). Suppression of adult neurogenesis with temozolomide (TMZ), caused learning deficits in the reference memory version of the water maze task(186). In this task, mice were tested in the hidden platform version of the Morris water maze for 6 trials per day for 5 days, with a reversal of platform location on day 4. Mice with suppressed neurogenesis presented deficits in the search of the new position of the hidden platform (reversal learning) on day 4, accompanied by deficits in the production of LTP from newborn neurons(186). This study suggests that new neurons are particularly important for adding flexibility to some hippocampus-dependent parameters of learning(186). In another study, ablation of neurogenesis induced impairments in spatial relational memory, which further supports the notion that newborn neurons are implicated in cognitive flexibility(187). On the contrary, aspects with less complex forms of spatial learning remained unaltered, further indicating that adult born neurons in the DG are necessary for complex forms of hippocampus dependent learning(149). A new theory proposes that newborn neurons inhibit the sparse activity of dentate granule neurons, thus allowing new distinct memories to form faster(149).

1.7.4 Hippocampal neurogenesis in mood and anxiety and the role of HPA axis.

Studies in humans and rodents have implicated hippocampal neurogenesis in the pathogenesis of depression and anxiety disorders(188–190). Chronic stress in rodents negatively regulates hippocampal neurogenesis with deleterious effects on behaviour, while the use of anti-depressants alleviate the behavioural outcome through regulation of neurogenesis process(190). In patients suffering from depression, the hippocampal volume is decreased, while post-mortem analysis of brains from depressed patients show significant decrease in the markers of neurogenesis(189). These findings led the neuroscience

community to propose the neurogenic theory of depression(191). The neurogenic theory of depression suggest that dysregulation of adult hippocampal neurogenesis triggers depression while its restoration through conventional anti-depressant therapy facilitates recovery(191).

In animal models, chronic stressors cause anxiety and depressive-like behaviors and impairs adult hippocampal neurogenesis(190). These stressors include chronic social defeat(192), unpredictable chronic mild stress(193–195), chronic immobilization(196), prenatal stress(196), early life stress(197) and corticosterone administration(192). In non-human primates, social stress induces depressive and anxiety-like behaviors, accompanied with decreased neurogenesis(198). Most rodent studies have reported that reducing or enhancing neurogenesis modulates anxiety-like and depressive-like behaviors only in response to stress, and that neurogenesis does not modulate these behaviors if stress is absent(149,190). Despite that, there are studies suggesting that even without prior stress, diminished neurogenesis in the hippocampus increases anxiety-like behaviour(199,200). Chronic stressors affect not only the process of hippocampal neurogenesis but also the formation of hippocampal information processing, therefore, altering the flow of information to downstream brain regions such as amygdala and hypothalamus, known for their regulatory role in anxiety and neuroendocrine function(149).

Stress activates the HPA axis with the subsequent release of glucocorticoids. Glucocorticoids are known to exert negative effects on adult hippocampal neurogenesis. In animal models, chronic treatment with corticosterone downregulate hippocampal neurogenesis and causes depressive and anxiety-like behaviors, which are reversed through adrenalectomy(200–202). The negative effects of glucocorticoids on neurogenesis have been recapitulated *in vitro* on adult human hippocampal neural stem cells(203,204). Despite the negative effects of glucocorticoids in neurogenesis process, neurogenesis in the hippocampus has been implicated in regulating body's response to and recovery from stress(205,206). Mice with impaired neurogenesis, exhibit deficits in HPA axis feedback inhibition and increased glucocorticoid surge after acute stress(205,206). These studies suggest that adult hippocampal neurogenesis maintains the hippocampal inhibitory control over the HPA axis, and impairments in neurogenesis exacerbate the glucocorticoid release, leading to further inhibition of neurogenesis. This is illustrated by the fact that neurogenesis is required for the recovery from anxiety, anhedonia and cognitive inflexibility, 4-6 weeks after exposure to a chronic stressor(207). The persistent hyper-activation of HPA axis leads to glucocorticoid abnormalities with long-term impairments in stress reactivity and mood regulation.

The role of adult hippocampal neurogenesis on mood and anxiety disorders is suggested to closely associate with the regulation of HPA axis and elegant cognitive processes such as pattern separation and cognitive flexibility(149). In humans, over-generalization is a hallmark of many anxiety disorders, possibly associated with impaired hippocampal neurogenesis(208,209). Recent studies have shown a correlation between depression scores and poor pattern separation(209). High depression scores were correlated with poor pattern separation among neutral stimuli, while enhanced pattern separation was observed among emotionally negative stimuli(209). These data were accompanied with decreased activity in

the DG and increased activity in the amygdala. During the encoding process of a fearful or stressful experience, a memory engram is formed in the DG. Once the stressor is over, cognitive flexibility is required to learn that the same context has become safe and is no longer associated with fearful or stressful stimuli (fear extinction). Some studies suggest that fear extinction requires cognitive flexibility-a neurogenesis mediated process(149). At the cellular level, it has been shown that adult-born neurons inhibit the sparse activity of mature dentate granule cells that previously encoded the memory engram of fear(149,210). Inhibition of the mature granule cells allows the erasure of the engram by facilitating the sparse activity of newborn neurons during the encoding of a new safe context. If hippocampal neurogenesis is compromised, the memory engram might not be sufficiently cleared from the DG, and the new safe context might not be discretely encoded(149). These deficits could contribute to imbalances in HPA axis activation and dysregulation of the limbic-anxiety-related circuits, with long-term effects on mood regulation(149). Therefore, increasing neurogenesis in patients suffering from depression and anxiety disorders might enhance reversal learning, fear extinction and cognitive flexibility(211).

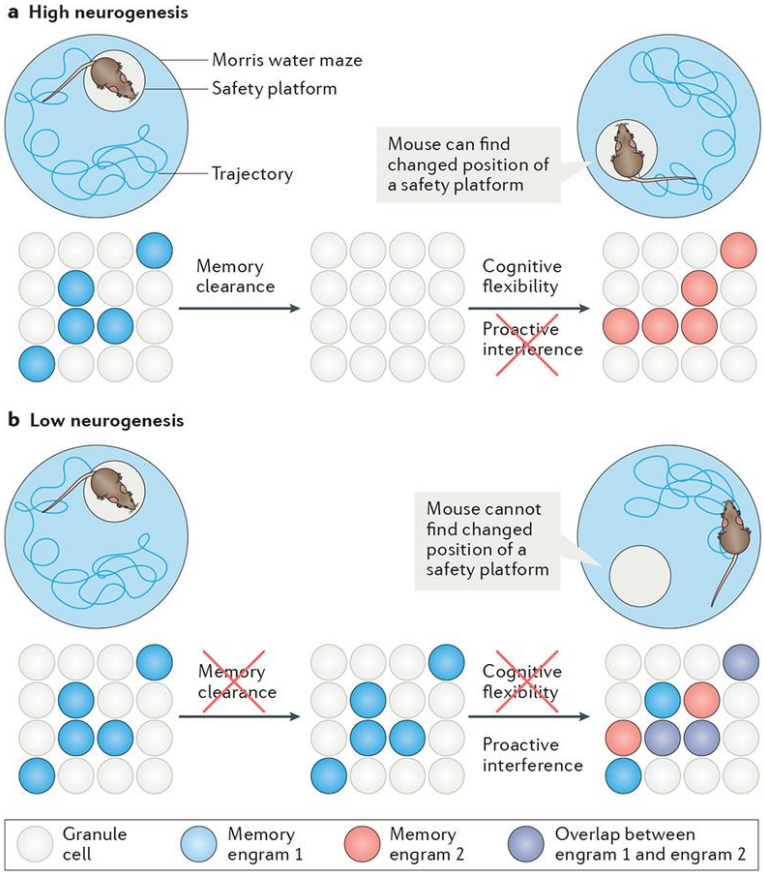

Figure 10. Adult hippocampal neurogenesis and cognitive flexibility. *Contextual memories are encoded by neural assembles in the DG. These cellular representations are called engrams or memory traces. (a) Increased neurogenesis facilitates the fast clearance and transmission of engrams to downstream processing structures. Memory clearance reduces proactive interference between previous context memories and the ability to accurately encode a novel context memory. This process facilitates reversal learning and cognitive flexibility to promote the finding of a new for example safety platform once the location has been changed. (b) Decreased neurogenesis may cause deficits in the clearance of previous context engrams. This might result in deficient encoding*

of contextual contingencies by neuronal ensembles. This may reduce cognitive flexibility and impair the ability to find the new location of a hidden safety platform. This figure was adapted from Christoph Anacker and René Hen, Nature Neuroscience, Volume 16, 335-346.

1.8 Regulation of Adult hippocampal neurogenesis.

Adult neurogenesis is a highly dynamic process and is regulated by a variety of intrinsic and extrinsic factors at several steps of the neurogenesis process. The first level of regulation occurs in the hippocampal network with neuronal activity affecting neurogenesis at several developmental stages (143). Adult hippocampal NSCs are responsive to gamma aminobutyric acid (GABA)(143). GABA promotes dendritic growth, synapse formation and survival of new neurons through CREB signaling(143). It has been shown that NMDAR signaling regulates survival of immature neurons in the SGZ(165). Moreover, NR2B is required for enhanced plasticity of new neurons in the DG during their critical period(165). Recently, it was reported that GABA released from interneurons in the hilus promoted division of RGLs-quiescent cells(135). GABA can also provide a trophic effect in the early development by promoting DNA synthesis in proliferating neurons and cell motility of immature neurons(135,141).

In the hippocampal niche, there is a number of morphogens who serve as niche signals to regulate the maintenance, activation, survival and fate choice of adult hippocampal progenitors(212). These morphogens include Notch, Shh, Wnts, and BMPs. In the SGZ, deletion of RBPj, a downstream mediator of all Notch receptors, activates radial glia-like cells to differentiate into transient amplifying progenitors, resulting in depletion of the quiescent pool of NSCs and subsequently loss of continuous neurogenesis(213). Shh signaling is activated in RGLs and is required for their establishment and maintenance in the SGZ(214). On the contrary, Wnt3 secreted by local astrocytes, promotes proliferation and neuronal fate commitment of neural precursors in the SGZ(215,216). BMPs promote glial differentiation and inhibit neural differentiation in the hippocampus(217–220). Blockade of BMP signaling in the hippocampal neural progenitors leads to their activation. However, long-term inhibition of BMP signaling results in depletion of hippocampal progenitors and loss of neurogenesis(217). Also, growth factors, neurotrophins such as BDNF, cytokines and hormones constitute important regulators of hippocampal neurogenesis(212). There are several neuromodulators such as glutamate, GABA, acetylocholine, dopamine and serotonin who directly modulate proliferation and survival of progenitors as well as maturation and migration of newborn neurons(212).

Intracellular components, such as, cell cycle regulators, transcription and epigenetic factors regulate adult hippocampal neurogenesis(212). Cell cycle regulators are key factors for the regulation of proliferation and fate choice of hippocampal NSCs(221). Inhibitors of the cell cycle plays a role in maintaining the quiescence of neural stem cells, and deletion of this factors such as p16, p21 and p53 leads to transient activation and subsequent depletion of the progenitor pool(221). Transcription factors are key players in the maintenance and renewal of progenitor cells(222). For example, the SRY-related HMG-box gene 2 (Sox2) is the major mediator of Notch signaling and contributes to the maintenance of the progenitor

pool in the SGZ(223). Shh is the direct target of Sox2 in neural progenitor cells and its deletion results in loss of hippocampal neurogenesis(224). In contrast, the transcription factors Prox1 and NeuroD are required for maturation and survival of newborn neurons in the DG(215,225). FoxOs are known to regulate intracellular signaling pathways and are required for the maintenance of adult neural progenitors(226). Also, the orphan nuclear receptor TLX is required for self-renewal and maintenance of neural progenitors in the SGZ(222). Epigenetic factors are key components of cell cycle regulation, gene expression and differentiation process of NSCs(212). For example, the Methyl-CpG-binding domain protein 1 (Mbd1) suppresses the expression of FGF-2 and several miRNAs to control the equilibrium between proliferation and differentiation process of hippocampal NSCs(227).

One of the most important aspects of adult hippocampal neurogenesis is its sensitivity to extrinsic stimuli either physiological or pathological(188). Hippocampal neurogenesis rapidly responds to stimuli coming from the periphery in every stage of the neurogenic process. Physical exercise, environmental enrichment and learning are among the physiological stimuli that affect neurogenesis positively(163). For example, physical exercise is known to increase cell proliferation while environmental enrichment promotes the survival of newborn neurons(163). Learning modulates hippocampal neurogenesis in a complex yet specific fashion. It has been shown that adult hippocampal neurogenesis is affected by learning tasks that are hippocampus dependent(160). Hippocampus dependent learning regulates mainly the survival of new neurons(160).

Pathological stimuli such as stroke, seizures, neuroinflammation, peripheral inflammation, stress and ageing affects adult hippocampal neurogenesis(188). Stroke induces cell proliferation and migration of new neurons to infarct sites(228). Seizures in experimental models induces proliferation of progenitor cells in the SGZ and alter the migration pattern of new neurons in the GCL of the DG(129,228). Neuroinflammation and peripheral inflammation negatively affects proliferation and differentiation of new neurons in the DG, mainly via activation of microglia cells(229). Chronic stress and ageing downregulate the number of proliferating progenitors as well as the number of newborn neurons in the region of hippocampus(174). In the next session we focus on the immune influence on adult hippocampal neurogenesis.

Table 1. Signals regulating adult hippocampal neurogenesis

	Stages	Regulators
Transcription factors	Type 1	**Maintenance of RGLs** (REST, Sox2, Hes5, FoxO, NFIX, NFIB) **Activation of RGLs** (Asc11)
	Type 2a	**Maintenance of NPs** (Sox2,TLX1, REST) **Differentiation of NPs** (Asc11)
	Type 2b	**Differentiation into intermediate progenitors** (Tbr2) **Neuronal differentiation** (Neurog2)
	Neuroblasts	**Neuronal differentiation** (NeuroD1)
	Immature Neurons	**Neuronal maturation** (Prox1, CREB, Klf9)
Neurotransmitters	Type 1	**Activation of RGLs** (GABA, Glutamate)
	Type 2a/2b and Neuroblasts	**Proliferation of NPs** (GABA, Dopamine, Serotonin, Norepinephrine, Acetylcholine) **Inhibition of proliferation** (opioids)
	Immature Neurons	**Activation** (GABA, Glutamate, Acetylcholine, Dopamine)
Secreted factors **(Morphogens, cytokines, growth factors etc)**	Type 1	**Maintenance of RGLs** (BMPs, VEGF, Shh) **Proliferation of RGLs/NPs** (IGF2)
	Type 2a/2b	**Proliferation of NPs** (FGF2, IGF-2, EGF, ERK5, estrogen) **Differentiation** (Wnt, IGF-1, VEGF, BDNF/NT-3,BMPs,) **Inhibition of proliferation** (cortisol, opioids, ApoE4)
	Neuroblasts and immature neurons	**Maturation** (Wnt/PCP, BDNF/NT-3, TIMP2) **Inhibition of proliferation** (CCL11, β2M)
Adhesion molecules	Type 1, 2a and 2b	**Maintenance of RGLs** (notch) **Inhibition of proliferation** (integrin) **Differentiation** (Eph-Ephrin)

	Neuroblasts and immature neurons	**Migration and synaptogenesis** (Semaphorin/Plexin) **Inhibition of proliferation** Tenasin-R
Epigenetic modifiers	Type 1, 2a and 2b	**Proliferation of RGLs/NPs** (GADD45b,TET1, miR-137, miR-17-92, Nup153, **Differentiation of RGLs/NPs** (MBD1,HDAC1, HDAC2, MeCP2, miR-184, miR-199
	Immature Neurons	**Synaptogenesis** (MeCP2, HDAC2) **Neuronal migration/dendritic growth** (miR-19, miR-132)

Table 2. Effects of extrinsic stimuli on adult hippocampal neurogenesis.

Experience	Radial glial stem cells (Type 1)	Amplifying progenitor cells (Type 2)	Neuroblasts (Type 3)	Cell survival	Dendritic Maturation	Axons
Exercise	↑	↑	↑	↑	↑	↑
Enrichment	↑	↑	↑	↑	↑	N/A
Stress	↓	↓	↓	↓	↓	N/A
Aging	↓	↓	↓	↓	↓	N/A
Antidepressant drugs	↔	↑	↑	↔	↑ or ↓	N/A
Stroke	↑	↑	↑	↑	↑	↑
Seizure	↑	↑	↑	↑	↑	↑
Brain injury	↑	↑	↑	↑	↑	N/A

1.8.1 Immune system and adult hippocampal neurogenesis.

For many decades the brain was considered as an immunologically privileged organ and there was the notion that cross talk between the immune system was unlikely due to the existence of BBB. But recently this notion has changed due to the discovery of the CNS lymphatic system that drains immune cells from the cerebral spinal fluid (CSF) to the lymph nodes(230,231). A plethora of studies report a profound capacity of the CNS to respond immunologically to injuries, infections and degeneration through the brain immune components such as microglia cells and astrocytes (232). Hippocampal neurogenesis is tightly regulated by the immune compartments of the brain as well as by circulating immune cells and cytokines travelling through the bloodstream in the brain(232).

The hippocampal niche is highly vascularized and the progenitor cells are in close proximity to blood vessels(233,234). This anatomical evidence suggests that the hippocampal NSCs receives extrinsic cues via circulating and endothelium derived factors(233). These factors affect proliferation, survival, differentiation and maturation processes. For example, the vascular endothelial factor VEGF stimulates proliferation of progenitors and exert neurogenic and angiogenic properties, suggesting that there is a direct link between the blood system and the brain(235). Also, following voluntary running the levels of circulating VEGF are upregulated and neurogenesis is increased(235). Interestingly, blockade of circulating VEFG via antibodies prevents the run-induced increase in proliferation of progenitor cells in the hippocampus further suggesting that a reciprocal communication exist between the periphery and the brain(235). In the hippocampal niche, cells with immune properties regulate neurogenesis under physiological and pathological conditions(232). Here, we focus on microglia cells and astrocytes as regulators of neurogenesis process in the hippocampus.

1.8.2 Microglia regulation of adult hippocampal neurogenesis

Microglia are the CNS resident myeloid cells and are uniformly arranged throughout the brain and constitute approximately 10-15 % of the total brain cells(236,237). During embryonic development, microglia progenitors originate from primitive myeloid precursors in peripheral mesodermal tissue(238). The microglia progenitors migrate from the extraembryonic yolk sac into the CNS(237). After the embryonic period, microglia maintenance is considered exclusively dependent on self-renewal of the CNS-resident microglia(237). Microglia cells are constantly modulated by various stimuli in their surrounding micro-environment(239). Under healthy conditions, microglia are in a "surveying" state in which they thoroughly scanning the surrounding environment to perceive activating or inhibitory signals(239). Microglia cells under basal conditions present a small cell body with thin, long and branched processes(240). Upon sensing environmental changes, such as, microbial invasion, cell injury or death, changes in pH milieu, alterations in the composition and integrity of extracellular matrix or the presence of inflammatory molecules, such as, cytokines and chemokines, microglia cells are activated(240). During activation microglia can be divided into two phenotypes: the classical M1 and the alternative M2 activation/deactivation state(240). The classical M1 activation occurs when microglia encounter foreign antigens or sense inflammatory molecules(241). This type of activation refers to the first line of defense and facilitates the recruitment of systemic immune cells in the brain(240,241). On the contrary, the M2 state of activation which is referred as "neuroprotective" is activated to suppress the inflammatory response by producing and releasing anti-inflammatory cytokines, neurotrophins, as well as cytoactive factors involved in the repair and restriction of the damaged extracellular matrix in the brain(240,241). However, chronic activation of this state of microglia facilitate neuroinflammatory states in the brain(240). Despite this categorization of active states of microglia cells, the truth is that microglia exhibit a very heterogeneous population in the brain. For example, in neurodegenerative diseases the transition of microglia cell from the M1 to M2 state and the deactivation of microglia is neither temporally nor spatially clearly defined and there are even suggestions of a dysfunctional microglia phenotype following chronic activation(240).

In the healthy brain, microglia cells form an “immunological blood-brain barrier” between the brain parenchyma and the vascular system (242). Upon inflammatory stimuli or injury these cells are responsible for recruiting blood-born neutrophils and macrophages. Interestingly, activated microglia cells are able to protect neurons from systemic immune cells by engulfing them(242–245). The termination of a neuroinflammatory state in the brain requires the recruitment of systemic T cells specific to CNS antigens where they promote the termination of the neuroinflammatory response together with microglia cells acquired the M2 phenotype(246–248). These data suggest that microglia not only constitute the first line defense of the brain but are the key mediators with the peripheral immune system(249).

The neurogenic niche of hippocampus contains microglia and are increasingly recognized as key modulators of neurogenesis processes either by engulfing apoptotic NSCs or by affecting the maturation and migration pattern of the newborn neurons in the GCL of the DG(249). Indeed, *in vitro* studies were the first to show the beneficial role of microglia in neurogenesis process. In these pioneer studies, co-cultures of microglia cells with neural stem cells or conditioned media from microglia cells into culture of neural stem cells, promoted neurogenesis via the increase in the numbers of neuroblasts(250,251). These studies demonstrated that soluble factors released from microglia induces neuronal differentiation. Further studies showed that microglia exert a pro-neurogenic effect, mediated by the activation of mitogen activated protein (MAPK), phosphatidylinositol-3-kinase/Akt and delta-Notch signaling pathways(251). Interestingly, *in vivo* studies in rats exposed to an enriched environment not only induces neurogenesis(252) but also the number of hippocampal microglia cells(253). These microglia cells exhibited a neuroprotective phenotype expressing MHC II and IGF-1 which are known to induce neurogenesis(253). This effect was abolished when the rats were treated with minocycline (anti-inflammatory drug) which inhibited microglia functioning and subsequently neurogenesis(253). Although the mechanisms of minocycline induced inhibition of microglia is not fully addressed, it is suggested that suppresses the expression of the IL-1β-converting enzyme, of the synthase of nitric oxide, of the ecyclooxygenase-2 (COX-2), of the prostaglandin E2 as well as of p38 mitogen-activated protein kinase(248). In a pioneer study, *Choi et al*. reported that wild type derived neural stem cells co-cultured with microglia cells derived from mice with Presenillin 1 (PS1) variants exhibit impaired proliferation and neuronal lineage commitment, phenotypes that are recapitulated with mutant microglia for PS1 conditioned media in which altered levels of selected soluble signaling factors are detected(254). These findings suggest that soluble factors released by microglia play a key role in the modulation of hippocampal neurogenesis, suggesting the existence of non-cell-autonomous mechanisms that govern FAD-linked PS1-mediated impairments in adult hippocampal neurogenesis(254). *Vukovic et al*. recently showed that microglia regulate hippocampal neural progenitor in response to exercise and aging(255). In this study, the authors used the transgenic Csf1r-GFP mice to investigate whether hippocampal microglia directly influence the activation of neural precursor cells(255). They demonstrated that physical exercise increased the proliferation of hippocampal progenitor cells via the activation of microglia, effects that were abolished when microglia were ablated(255). Moreover, microglia from the hippocampus of exercised animals were able to activate progenitor cells when added to neural stem cell cultures from

non -exercised animals(255). The authors found that CX(3)CL1-CX(3)CR1 signaling mediates the observed effects of microglia to neurogenesis process. Intraparenchymal infusion of blocking antibody against the CX(3)CL1 receptor dramatically reduced the neurosphere formation in exercised mice(255). Moreover, in the blood of exercised mice the soluble form of CX(3)CL1 was increased, an effect that was not evident in the blood of aged mice. Lower plasma levels of CX(3)CL1 in aged mice was correlated with the natural decline in neural progenitor cell activity, a state that could be partially alleviated through removal of microglia(255). These data suggest that microglia cells exert a dual and opposing influence on neural progenitor cell activity within the DG through CX(3)CL1-CX(3)CR1 signaling.

Apart from the effects of microglia on progenitor cells proliferation, differentiation and survival in the hippocampal niche, microglia also regulate neurogenesis through the phagocytosis of adult born cells in the neurogenic niche(256). The majority of newborn cells undergo apoptosis in the first 1 to 4 days of their life, during the transition from amplifying progenitors to neuroblasts(256). These apoptotic cells are rapidly cleared out through phagocytosis from microglia cells residing in the SGZ. *Sierra et al.* found that phagocytosis is mediated by unchallenged microglia independently of age or the presence of inflammation(256). These findings suggest that the homeostatic activity of microglia cells in the neurogenic niche regulate the neurogenesis cascade(256). Additionally, microglia has been implicated in synaptic pruning by phagocytic engulfment of synapses on mature neurons in the healthy brain, suggesting a role of microglia in the later stages of neurogenesis, such as, during the periods of synaptic assembly, stability and transmission(257,258).

Moreover, *Mosher et al.* demonstrated that newborn neurons modulate the function of microglia in the DG, by secreting various factors, suggesting that the communication between microglia and newborn neurons is bidirectional(259). They found that microglia cells are densely populated in the neurogenic niche and in great proximity with neural stem cells(259). NSCs secrete several immunomodulatory proteins such as the tissue inhibitor of metalloproteinase type-1, VEGF and haptoglobin, which are known to modulate microglia function(259). In the DG interneurons, release of the neuropeptide VIP (vasoactive intestinal peptide) stimulate microglia cells to produce IL-4, which enhances neurogenesis and survival in hippocampal cultures. This effect is mediated by the binding of VIP on microglia VPAC1 receptors, suggesting the existence of a neuroimmune-neurogenic pathway in which neuronal activity modulate microglia activity and subsequently neurogenesis(260).

1.8.3 Microglia regulate adult hippocampal neurogenesis during inflammatory conditions.

During inflammatory or pathological conditions, microglia are activated and release inflammatory mediators that can be either neurotoxic or neuroprotective(249). Classical activated microglia commonly exert negative influence on adult neurogenesis. It has been shown that cranial irradiation or administration of high doses of bacterial lipopolysaccharide (LPS) inhibit neurogenesis in the DG(261–263). LPS induces the expression of mediators involved in inflammatory response such as the nuclear factor kappa B (NF-Kb) and

components of the MAPK pathway, including p38 and c-Jun(264–266). NH2-terminal kinase (JNK) activation leads to the production of cytokines such as IL-6 and TNF-α which disrupt neurogenesis process(264,266). In the presence of IL-6 adult hippocampal neurogenesis is reduced and its inhibition restore this deficit(262). IL-6 is involved in the regulation of synaptic plasticity, LTP and memory(242). Tumor necrosis factors such as TNF-α, are a family of cytokines known to cause apoptosis. TNF-α has an anti-neurogenic effect on NSCs and is upregulated in a number of neurodegenerative disorders. TNF-α signaling affects adult hippocampal neurogenesis in a TNF-subtype receptor specific manner, with signaling through the TNFR1 to exert negative and TNFR2 to exert positive regulation of hippocampal neurogenesis(242). Moreover, LPS is known to induce TLR4 receptor with subsequent activation of signaling pathways leading to the production of pro-inflammatory cytokines(267).

The pioneer study of *Monje et al.* showed that intraperitoneal administration of LPS induced a profound increase of activated microglia cells in the DG(262). This activation was accompanied with a decrease in proliferation and neurogenesis process(262). Co-culture of activated microglia but not resting microglia with neural stem cells derived from hippocampus induced a profound decrease of DCX^+ cells(262). LPS alone did not have any effect on proliferation and neurogenesis processes, suggesting that the effects on neurogenesis are mediated by the active microglia(262). Indeed, adding conditioned medium from activated microglia cells into neural stem cells culture decreased neurogenesis(262). The authors determined TNF-a and IL-6 as the negative regulators of hippocampal neural stem cells. It is known that signaling via gp130, the co-receptor of IL-6 stimulates the Notch1 pathway and increase the expression of the mammalian homolog of hairy-enhancer-of-slit (Hes1) transcription factor, an antagonist of the pro-neural basic helix-loop-helix (bHLH) genes and hippocampal neurogenesis during development(249). Treatment with recombinant IL-6 or conditioned medium from activated microglia induced a dramatic decrease in Hes1 mRNA expression(249). Inflammatory blockade with indomethacin (a nonsteroidal anti-inflammatory drug) decreased microglia activation and restored neurogenesis(268). This study suggest that inflammatory blockade attenuated the release of pro-inflammatory cytokines, the activation of HPA axis and the apoptosis of newborn cells(261). Accordingly, *Ekdahl et al.* provided *in vivo* evidence that systemic LPS-induced CNS inflammation impaired neurogenesis process in the healthy hippocampus, while induced neurogenesis in response to seizure(268). Activated microglia were found in close proximity to newly born neurons and were negatively correlated with the number of survived new granule neurons in the DG(261). Treatment with minocycline restored hippocampal neurogenesis, further indicating that the detrimental effects of LPS -induced inflammation was mediated by activated microglia(268). Apart from the effects on neurogenesis, *Fujoka and Akema* reported that 5 hours after a single intraperitoneal (ip) injection of LPS the expression of the proliferation marker pH3 (serine 10 phosphorylated histone H3) was decreased in the SGZ of the DG(269). The same results were found 24 hours after labelling cells with BrdU(269).

Apart from the effects of inflammation on proliferation, survival and cell death of hippocampal neural stem cells, inflammation exert negative effects on migration and

integration of new born neurons in the circuitry of the DG(270). *Jakubs et al*. was the first to demonstrate that neuroinflammation induced by intrahippocampal infusion of LPS regulates the functional integration of new neurons in the circuitry of the hippocampus(271). LPS induced activation of microglia while neurons born one week after LPS infusion showed no changes in dendritic tree morphology, location within the GCL, intrinsic membrane potential or axon projection(271). Whereas, the persistent and chronic neuroinflammation altered the synaptic input of both pre-existing and newborn neurons(271). Both mature and newly formed neurons showed an overall increase in the frequency of spontaneous excitatory postsynaptic current (EPSC), compared to granule cells in control animals, indicating that the existence of an inflammatory environment causes an increase in excitatory activation(271). Despite the increased excitatory input, only the new neurons born one week after LPS administration showed an enhanced inhibitory input(271). Interestingly, the time between the spontaneous inhibitory postsynaptic current (IPSC) was shorter in new neurons compared to mature granule cell neurons(271). Moreover, newborn cells had higher density of inhibitory synapses on their distal dendrites compared to mature granule neurons. These data indicate that neurons that mature in an inflammatory environment have increased inhibitory input from synapses that may potentially alter new neurons formation(270).

Another fundamental study showed that chronic inflammation reduces new neuron participation in hippocampal processing of contextual information(229). In this study, rats received intracerebroventricular infusions of LPS or cerebral spinal fluid (CSF) for 28 days and then received five BrdU injections to label the dividing cells(229). Two months after the last BrdU injection rats were placed in a novel environment and allowed to explore for 5 minutes and sacrificed 30 minutes later, in order to assess the expression of the plasticity related immediate early gene *Arc* (activity regulated cytoskeleton-associated protein) in mature and new neurons in the GCL(229). Chronic LPS induced activation of microglia in the hippocampus as evidenced by increased MHC II expression two months after treatment(270). Interestingly, no differences were found in the number of BrdU-expressing new neurons in the GCL(270). This discrepancy might be due to the use of a lower dose of LPS than prior studies did. As it was expected, 30 minutes after exploring the novel environment control animals exhibited a greater proportion of new neurons expressing Arc compared to mature neurons, suggesting that inflammatory environment increased the recruitment of new neurons(229). However, in the LPS-treated animals there was no difference in Arc expression between new and mature neurons, indicating that chronic inflammation attenuated the recruitment of new neurons into hippocampal networks(229). These findings in conjunction with the aforementioned study of *Jakubs et al.* suggesting that inflammation alters the unique cellular processes that new neurons display during development, making them less able to fully integrate and function in pre-existing hippocampal networks(270).

As we thoroughly described above, classical activated microglia exert negative influence on adult hippocampal neurogenesis process. On the contrary, alternative activated microglia exert neuroprotective effects on neurogenesis process through the secretion of anti-inflammatory cytokines(272). The alternative phenotype of microglia is characterized by

expression of MHC II, arginase 1 (AG1), mannose receptor (MRC1), peroxisome proliferation activation receptor gamma (PPAR-c), Ym1 (Chitinase 3-like 3) and found inflammatory zone 1 (FIZZ1)(273). Further, the alternatively activated microglia express the anti-inflammatory cytokines interleukin-10 (IL-10), transforming growth factor b (TGF-b), and growth factors such as insulin-like growth factor (IGF), neural growth factor (NGF), and brain derived neural growth factor (BDNF)(273). The expression of the alternative phenotype often follows an inflammatory response, potentially making the alternative phenotype a compensatory response to aid in healing(274,275).

Evidence suggests that anti-inflammatory cytokines support neurogenesis(272). *Battista et al.* (2006) found that TGF-b facilitates the observed increase in hippocampal neurogenesis following adrenalectomy (ADX). ADX has been reported to increase proliferation and neuronal differentiation(276). Moreover, inhibition of TGF-b with an antibody had no effect on the ADX-induced increase in proliferation, while reduced neuronal differentiation, suggesting a role in the differentiation process(276). Also, it has been shown that conditioned medium from microglia stimulated with IL-4 increases the number of progenitors in adult NSCs cultures(263). Additionally, more cells were found to be DCX^+ when co-cultured with microglia stimulated with IL-4 compared to control cells that were cultured with un-stimulated microglia(263). Stimulation of microglial cells with IL-4 increased microglia expression of IGF-1 that is known to support neurogenesis(277). In agreement with the above studies, a recent paper reported that co-culturing NPCs with IL-4 stimulated microglia enhanced neuronal differentiation. The same phenomenon was observed with IL-10. IL-10 stimulated microglia enhanced NPCs proliferation, but had no effects on differentiation(278). Further, *Cacci et al.* found that microglia releasing IL-10 were supportive of neuronal differentiation and new cell survival(275). These data indicate that anti-inflammatory cytokines produced by an alternative activated microglia support survival and enhance the production of new neurons.

Additionally, the study by *Kiyota et al.* showed that overexpression of the anti-inflammatory cytokine IL-10 attenuates the reduction of neurogenesis in an Alzheimer's mouse model(278). *In vivo* overexpression of IL-10 in the hippocampus of APP + PS1 mice increased the number of new cells and the number of DCX^+ and $BrdU^+$ /$NeuN^+$ cells(278). It has been proposed that IL-10 levels within the hippocampus reduce the activation of microglia towards the M1 type and increase the expression of the M2-type that support neurogenesis. Collectively, the above findings indicate that the efficient balance of pro- and anti-inflammatory activity within the brain may have beneficial effects on neural plasticity in the diseased brain.

1.8.4 The role of astrocytes on hippocampal Neural Stem Cells (NSCs)

The role of astrocytes in the function of the brain is well defined. Astrocytes mainly provide metabolic support to neurons(279), water homeostasis(280), exchanging information between neurons and blood vessels to coordinate oxygen and glucose delivery, control of extracellular ion flux and antioxidant support by glutathione release(281), the uptake and clearance of neurotransmitters(282) and the maintenance of the integrity of BBB(283). A less stated function of astrocytes are their ability to exert immune-like properties such as

phagocytosis and antigen presenting capacity(284). Astrocytes are known to express major histocompatibility complex class I (MHC I) and MHCII antigens upon stimulation with interferon gamma (IFN-γ) *in vitro*, which can lead to T cell activation(285). Also, astrocytes are sensitive to the effects of cytokines and in the presence of an injury are activated, forming a glial scar that is thought to be beneficial for the surrounding neurons(286). Following injury or infection astrocytes produce cytokines and chemokines that mediate innate immune function such as the recruitment of microglia cells and monocytes(286).

Astrocytes play also a role in neural stem cell physiology. They release soluble factors which influence distinct stages of adult neurogenesis including proliferation(287), neuronal differentiation(288), neuronal survival(289) and synaptic integration(290). Interestingly, CNS injuries can produce reactive astrocytes with NSCs properties(291). In contrast, activated astrocytes during neurodegeneration do not exhibit stem cell properties. In addition, blocking the reactive gliosis leads to improved integration of neural precursor cells in the hippocampus(292).

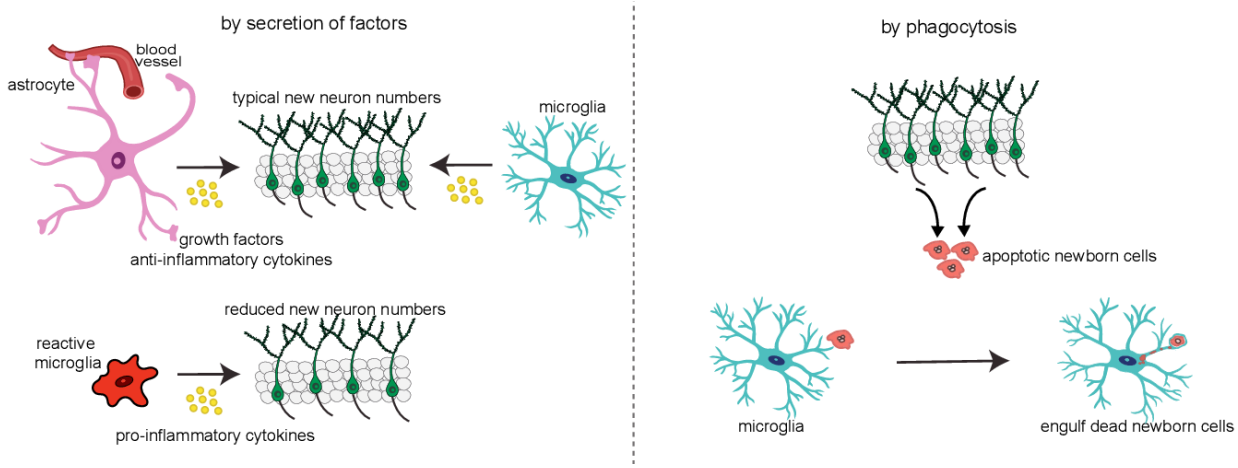

Figure 11. Effects of glia on hippocampal neurogenesis. *Astrocytes and microglia secrete growth factors and anti-inflammatory cytokines that promote adult neurogenesis. Under pathological conditions, activated microglia produce pro-inflammatory cytokines that impair adult neurogenesis. Microglia participate in adult neurogenesis by phagocytosing apoptotic newborn cells under physiological or pathological conditions. This figure was adapted by Cope and Gould, Cell Stem cell 24, 2019.*

1.9 Gut brain axis regulate adult hippocampal neurogenesis and microglia

Accumulating evidence support a causal link between neurogenesis process in the hippocampus and gut brain axis function(5). Alterations in gut microbiota or activation of the vagus nerve affect hippocampal neurogenesis directly or indirectly by modulating the function of microglia(5).

A recent study showed that prolong treatment with antibiotics decrease neurogenesis in the hippocampus(293). Colonization with probiotics and voluntary running rescued the negative effects of antibiotic treatment on neurogenesis(293). Interestingly, reconstitution with normal gut flora did not fully rescue neurogenesis in mice with antibiotic treatment. Probiotics and voluntary running increased the proportion of $Ly6C^{hi}$ monocytes in the brain.

Lack of this cell population, either by antibody treatment or through the use of CCr2 knockout mice, decreased neurogenesis. On the contrary, adoptive transfer of Ly6C^{hi} monocytes, rescued neurogenesis in the antibiotic-treated mice. Interestingly, microglia population was not affected by antibiotic treatment(293). Despite that, microglia and other immune cell populations are severely affected under germ-free (GF) conditions(294). In instance, microglia exhibit altered cell numbers and immature phenotype with deficits in immune responses(294). Defects or temporal eradication of gut microbiota induces defective microglia(294). On the other hand, reconstitution of the gut with a complex microbiota partially restores the defective microglia. The short-chain fatty acids were found to regulate microglia homeostasis as mice deficient for the receptor of SCFA, FFAR2 exhibited defective microglia(294). Collectively, these findings indicate that gut microbiota regulate microglia maturation and function, whereas microglia impairments can be rescued by complex microbiota. Interestingly, germ-free mice presented with anxiety-like behaviour and altered social interaction(295). In parallel, these mice displayed increased transcription levels of BDNF in the hippocampus(295). Moreover, neurogenesis is increased in germ-free mice(296), which is consistent with the increase in hippocampal BDNF. Also, the observation that germ-free mice display higher motor activity may also account for an increase in neurogenesis(297).

We previously discussed that the main communication root of gut and the brain is mediated by the vagus nerve(5). Acute vagus nerve stimulation in rats for 3 hours induced an increase in proliferative NSCs of the hippocampus and this effect was evident for 3 weeks after treatment (298). It also induced long-lasting increases in the amount of neuroblasts(298). On the contrary, chronic vagus nerve stimulation did not increase proliferation or neuroblasts but increased the number of cells expressing BDNF as well as the dendritic complexity of neuroblasts(299). Chronic vagus nerve stimulation had no effect on behaviour in the forced swim or elevated plus-maze tests(299). These data indicate that vagus nerve stimulation exert direct and long-term effects on neurogenesis processes, but with no evidence of behavioural alterations associated with characteristic antidepressant or anxiolytic action. Conversely, subdiaphragmatic vagotomy decreased BDNF mRNA levels in every area of the hippocampus, reduced the number of proliferating cells, immature neurons as well as the number of survived and differentiated newborn neurons(299). Collectively, these data indicate that vagal nerve activity influences neurogenesis and transcriptional levels of BDNF in the adult hippocampus.

1.10 Innate immune memory of microglia

Immune memory has been longed considered as the ability of the mammalian adaptive immune system to retain memory for specific infectious agents, providing long-lasting protection against re-infections with the same pathogenic agent(300). However, in recent years, it has become clear that innate immune cells, including myeloid cells, are capable of retaining immune memory(300). Specifically, following a short-lived immunostimulatory event, a broad reprogramming of innate immune cells is elicited, resulting in enhanced production of pro-inflammatory molecules in response to a subsequent and temporally distant immunostimulatory event. This has been termed “trained innate immunity” or “innate immune memory”. The states of innate immune memory are manifested in two

major forms: a) immune “training” and immune “tolerance”, which refers to exacerbated or alleviated immune responses upon secondary stimulation, respectively through cytokine release(300). These two forms of innate immune memory can be elicited by heterologous stimuli, meaning stimuli different from the original insult(301). Immune training was originally used to describe cross-protection between infections. For example, it has been shown that *in vivo* incubation of the tuberculosis (TB) vaccine Bacillus-Calmette-Guerin (BCG), induces protection (immune training) against a subsequent infection of *Candida albicans* in animal models, as well as against infection with a yellow fever virus vaccine stain in humans(302). On the contrary, septic doses of LPS both in rodents and human subjects, elicits immune tolerance. Notably, immunosuppression in monocytes following sepsis can be alleviated through *ex vivo* treatment of monocytes with b-glucan, which mimics the training effects of BCG(302).

The molecular basis of innate immune memory in macrophages was found to be epigenetic reprogramming, in particular, epigenetic changes in various enhancers of immune-and metabolic-related genes(300,303). These epigenetic changes can modify the functional phenotype of innate immune cells, thus altering the inflammatory response, subsequently affecting pathology(300,303). In the periphery, immune training and tolerance contribute to the outcome of infections, where trained responses can be beneficial in response to pathogens, but at the same time can induce the exacerbation of peripheral pathologies, such as atherosclerosis, by facilitating the inflammatory response in atherosclerotic plaques(303). Therefore, innate immune memory has been considered to play a significant role in health and disease(303).

Recently, it has been shown that acute peripheral inflammatory stimuli can induce innate immune memory in the brain via epigenetic reprogramming of microglia cells, that in long-term shapes neuropathology(304). More specifically, a single intraperitoneal injection of LPS (0.5 mg/kg from *S.typhimurium*) caused immune training 1 day later, while repeated doses of LPS injections for 4 consecutive days induced immune tolerance, as evidenced from increased and suppressed production of several pro-and anti-inflammatory cytokines respectively(304). Furthermore, microglia-specific knockout of histone deacetylases-1 and 2 (Hdac1/2) or of Tak1 (an activator of NF-kB signaling) in mice abolished the effects of immune training, indicating for the first time that microglia are the main mediators of immune training in the brain(304). The long-term effects of immune training and tolerance were sufficient to modulate brain pathology in mouse models of stroke and Alzheimer’s disease (APP23 trangenic mice). Notably, a single i.p. LPS (immune training) dose 6 months before analysis in APP23 mice reduced the levels of the anti-inflammatory cytokine IL-10 to wild type levels, while repeated doses of i.p LPS (immune tolerance) reduced IL-1b concentrations to those of wild type mice(304). Concomitantly, immune training led to increased plaque load in the brain, while immune tolerance decreased it(304). Interestingly, these changes occurred despite the same levels of amyloid-b and microglia numbers in the brain. Moreover, purified microglia from wild type and APP transgenic mice 6 months after the last dose of LPS, showed epigenetic changes in active enhancers in pathways reflecting significant parallels in gene expression and cell function. For example, consistently

enhanced pathways were the hypoxia-inducible factor-1a (HIF-1a) pathway in trained microglia, and the Rap1 signaling pathway in tolerant microglia from APP transgenic animals. HIF-1a has previously been implicated in mediating innate immune training in macrophages *in vitro* and *in vivo*, while immune tolerance increased phagocytosis of microglia *in vitro*. Moreover, microglia isolated from both wild type and APP transgenic mice treated with either 1 or 4 doses of LPS 6 months earlier, showed upregulation of microglia homeostatic genes such as *Tmem119*, *P2ry12* and *Cx3cr1* together with most DAM associated genes in one gene module(304). The pathways enriched in this module were specific to lysosome, phagosome and oxidative phosphorylation. Another study has shown that a single LPS challenge (0.25 mg/kg) produced significant and lasting suppression of LPS-induce IL-1b expression in murine microglia *ex vivo* and *in vivo.* Epigenetic changes were found in the IL-1b promoter, where H3K9/K14 acetylation and H3K4 tri-methylation led to robust transcriptional activation after primary LPS exposure, but significantly less after a second exposure. In line, there was robust H3K9me2 methylation at IL-1b promoter site in all animals receiving the preconditioning LPS stimulus, suggesting the occurrence of transcriptional repression(304).

Innate immune training has been recently shown to affect stem cell biology. Recently, *Mitroulis et al.* investigated the metabolomics and transcriptomic effects of innate immune training on hematopoietic stem cells. Specifically, the study showed that injection of b-glucan upregulated various pathways associated with cell proliferation, including cell cycle genes, cholesterol biosynthesis and glycolysis(305). These changes were accompanied by expansion of myeloid hematopoietic progenitors(305). This myeloid expansion persisted for up to a month, and transplantation of hematopoietic stem cells from b-glucan trained mice yielded myeloid-skewed immune populations in untrained mice(305). These effects were rescued by blockade of either glycolysis or IL-1b signaling which prevented the myeloid expansion. Interestingly, upon LPS challenge, b-glucan-trained mice made more myeloid progenitors and accumulate less DNA damage(305). Also, the negative effects of chemotherapy in mortality and DNA damage were reduced in b-glycan-trained mice(305). Another study, has also shown that vaccination of mice with BCG, induced proliferation of hematopoietic progenitors(306). When the authors depleted T cells from bone marrow of mice trained with BCG and transplanted into irradiated recipients, mice exhibited better responses against *Mycobacterium tuberculosis*(306). Also, macrophages derived from trained bone marrow have better responses against *Mycobacterium tuberculosis in vitro.* The authors identified that all these phenotypes were dependent on IFN signalling(306). In a very sophisticated study, the authors used a model of metabolic syndrome which induced auto-inflammation by feeding Ldlr-deficient mice a high-fat Western diet (WD)(307). After 4 weeks, they found increased levels of circulating inflammatory monocytes, increased proliferation of myeloid lineage precursors and differentiated cells as well as more activated monocytes(307). Interestingly, when WD-fed mice were switched back to conventional diet (CD) for 4 weeks, the myeloid progenitors exhibit a transcriptional profile more similar to that of WD than of CD while they had a distinct pattern of chromatin accessibility and monocytes remained activated(307). These monocytes when in culture secreted higher levels of inflammatory cytokines in response to Toll-like receptors agonists than monocytes

derived from CD(307). In the molecular level, Nlrpe3-dependent inflammasome activation and IL-1b production were required for the inflammatory effects of WD(307). Although, this study did not investigate the effects of secondary responses it suggests that myeloid progenitors and differentiated cells can exhibit trained immunity phenotypes in response to an autoinflammatory state. Overall, these studies suggest that trained immunity can affect stem cell biology and provide proof of principle that immune training can influence subsequent immune responses *in vivo*.

1.11 Aim of the study

The aim of this study was the characterization of the effects of experimental acute and chronic colitis, induced by administration of dextran sodium sulfate (DSS) in mice, on adult hippocampal neurogenesis and microglia activation. Epidemiological studies suggest that the prevalence of depression and anxiety is much higher in IBD patients as compared to the general population and correlates positively with the severity of the disease. There are few functional magnetic resonance (fMRI) studies that identified structural changes or alterations in the activation of cortical and limbic circuits involved in emotional processing and cognitive function in IBD patients. These studies indicate that there is a biological basis in the mechanisms that affects the functionality of brain circuits at the cellular and molecular level. The mechanisms by which depression and cognitive dysfunction occur in IBD patients are still unknown but a plethora of new studies suggest that an inflammatory environment in the gut leads to behavioural abnormalities characteristic of depressive and anxiety-like symptoms in a bottom-up manner.

We first determined the effects of acute and chronic DSS colitis on the various stages of adult hippocampal neurogenesis. Our results showed that 7 days of DSS administration induces activation of the quiescent pool of NSCs ($GFAP^+/Ki67^+$), along with increases in fast proliferating progenitor cells ($Sox2^+$) and neuroblasts (DCX^+). Then, we focused on cell cycle kinetics of proliferating progenitors as it is known that regulation of the cell cycle length during systemic inflammation affects the differentiation process of NSCs, restraining progenitors from proliferation and protecting them against inflammation-induced apoptosis. Our findings showed that the duration of the S-phase of proliferating progenitors in the DG of mice with DSS colitis is significantly reduced. This effect seems to be cell type-specific, as only Type 2a and Type 3 cells exhibited reduced numbers of $BrdU^+$ cells in the DG during acute DSS colitis.

Chronic DSS colitis recapitulates features of human Ulcerative colitis. As the outcome of hippocampal neurogenesis is the production of new neurons that functionally integrate in the hippocampal circuitry, we hypothesized that chronic intestinal inflammation may alter the survival of newborn neurons. In our study this was not evident as the number of newborn neurons were similar to the control group. However, when we examined the migration of newborn neurons under chronic intestinal inflammation we found a lower migration pattern. Thus, we hypothesized that the migration deficits in newborn neurons of the hippocampus observed in the context of chronic intestinal inflammation affected their integration and, as such, induced functional impairment. Indeed, previous studies using the immediate early

gene *Arc* as a marker of neuronal activity, showed that 4-6-week old adult newborn neurons are less recruited in the DG, under neuroinflammatory conditions in response to a novel spatial environment. To explore this possibility, we utilized a well-established behavioral paradigm and assessed whether 7 week- old newborn neurons were functionally integrated in the DG circuitry of mice with chronic DSS colitis. Our findings provide evidence that chronic intestinal inflammation induces a significant decrease in Arc expression in both mature and newborn neurons in the DG, as compared to controls, a finding that may be associated with cognitive dysfunction, as mentioned above. Taken together, these data propose that intestinal inflammation affects neurogenic processes in the hippocampus, alters neuronal integration patterns and dysregulates neuronal function in response to a novel spatial environment.

During a peripheral inflammatory insult, microglia in the brain responds by altering their activation status. Microglia communicate bi-directionally with NSCs in the hippocampus and are integral parts of the neurogenic niche. Given their extensive role in inflammatory response and adult NSCs biology we next evaluated their activation status during acute and chronic DSS colitis. Briefly, to explore early activation of innate immune cell responses in the hippocampus, we chose an earlier time point during the course of acute DSS colitis (day 4), wherein signs of intestinal and systemic inflammation were already present. We initially confirmed that 4 days following DSS administration, the levels of myeloperoxidase (a marker of infiltrating macrophages) were increased in the colon, and the levels pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in the serum were increased. Moreover, we demonstrated that acute DSS colitis induced an increase in the percentages of microglia with an M1-like phenotype ($CD45^{low/-}CD11b^{+}NOS2^{+}$) in the hippocampus, along with enhanced infiltration of inflammatory myeloid cells ($CD45^{high/+}CD11b^{+}Ly6C_{+}$) from the periphery (both on days 4 and 7 post DSS administration). Moreover, the levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were elevated in the serum and hippocampus at both time points. Interestingly, we observed, for the first time to our knowledge, an infiltration of M1-like macrophages in the hippocampus in the context of acute DSS colitis, an effect that seems to be IBD-dependent, as previous studies using intraperitoneal LPS injection did not detect macrophage infiltration in the brain. In contrast, during the chronic phase of DSS colitis, our findings revealed that microglia acquired an M2-like phenotype ($CD45^{low/-}CD11b^{+}Arg\text{-}1^{+}$), while the percentages of inflammatory myeloid cells and M1-like microglia were decreased. In parallel, the levels of the sera and hippocampal pro-inflammatory cytokines were decreased, while the production of the anti-inflammatory cytokine IL-10 was enhanced.

These data were further confirmed with immunohistochemical analysis where microglia stained with the microglia marker Iba-1. We found increased numbers of microglia in the neurogenic niche of the hippocampus in mice with acute and chronic DSS colitis. Further morphological analysis confirmed the above immunophenotypic analyses of microglia. Also, the protein levels of the ^{pser9}GSK3b kinase were elevated during acute DSS colitis, which confirm the above anti-inflammatory phenotype of microglia, as in this form, the kinase exerts anti-inflammatory properties. In parallel, the transcriptional levels of Arginase

1 and BDNF were elevated during acute DSS colitis, further confirming the aforementioned results.

Experimental DSS colitis is the gold standard for studying the human disease in rodents, particularly when the focus is on the short- and long- term effects of epithelial barrier disruption and the associated chronic inflammation. As such, our findings might have important clinical implications, as they propose that DG dysfunction could be a valuable indicator of the vulnerability of IBD patients in the development of cognitive deficits and depression. If this hypothesis is supported by translational studies, the development of targeted treatments that can alter neurogenesis, and mood and cognition disturbances, such as, anti-depressants or physical exercise, should be considered in the therapeutic management of IBD patients.

2. Materials and Methods

2.1 Animals

Wild-type male mice of C57BL/6 genetic background weighing 24 to 26 g and 10-11 weeks old were used. The animals were housed in plastic cages under controlled conditions (temperature 22 °C; 12-h light/dark cycle; lights on at 7 am) and were given free access to standard laboratory pellet formula and tap water. Animals were habituated to housing conditions for 2 weeks prior to the beginning of the experimental procedures. Daily inspection of cages for food spillage and monitoring of body weight and food intake were performed for the duration of the study. The experiments were approved by the competent veterinary authority of the prefectures of Athens, Greece, in accordance with the National Registration (Presidential Decree 56/2013) and with European Directive 63/2010.

2.2 Induction of acute and chronic DSS colitis

DSS induced- Acute colitis(83).

1. On day 1, mice were weighed and marked.
2. The drinking supply of the mouse cages were filled with DSS solution to the final concentration of 2.5 % w/v (MW= 36,000-50,000 kDa; MP Biomedicals). At least 5 ml DSS solution was calculated per mouse per day. In a biosafety hood, stock DSS solution was poured into 50 mL Falcon tubes (one needed per cage). Control mice received the same drinking water without DSS.
3. At day 3 the remaining DSS solution from the cage water bottles was discarded and refilled with DSS for another 2 days.
4. At day 4 the remaining DSS solution from the bottles were discarded and refilled with DSS solution.
5. On day 8 the remaining DSS solution was replaced by autoclaved water.
   Mice were weighted daily and the clinical progression of colitis was evaluated.

DSS induced- Chronic colitis (2& 3 cycles of DSS administration)(83).

6. The induction of chronic colitis is based on the administration of DSS for several cycles. The steps 1 to 4 of the Acute DSS colitis protocol were repeated on days 1-7 (1^{st} cycle of DSS treatment). The drinking supply of the mouse cages were filled with DSS solution to the final concentration of 2% w/v.
7. At day 8 the remaining DSS solution was replaced by autoclaved drinking water without DSS for 14 days.
8. The steps 2 to 4 from the Acute DSS colitis protocol were repeated on days 22-26 (2^{nd} cycle of DSS treatment). The drinking supply of the mouse cages were filled with DSS solution to the final concentration of 2% w/v.
9. At day 29 the remaining DSS solution was replaced with autoclaved water for 14 days.
10. The steps 2 to 4 from the Acute DSS colitis protocol were repeated on days 43-47 (3^{rd} cycle of DSS treatment). The drinking supply of the mouse cages were filled with DSS solution to the final concentration of 2% w/v.

11. On day 50 the remaining DSS solution was replaced with autoclaved water for 14 days.
    At several time points (on days 1-7, 9-14, 16-21, 21-28, 28-35, 43-49) of Chronic DSS- induced colitis mice were weighted and the clinical progression of colitis was evaluated.

2.3 Spatial Exploration

The animals were handled once daily for 49 days until the day before the experiment, when the animals remained undisturbed(308). The exploration environment was a square open box, 44x44 cm with 30-cm-high walls. The box was located in a 250 x 150 cm dimly lit room with several landmarks on the walls and ceiling(308). Each mouse was transported to the exploration room and placed in the center and left to explore freely the area for 5 minutes (1st exploration session). Then, mice were transferred to the home cage and remained undisturbed for 30 minutes. The second exploration session was performed identically to the first. Immediately, after, the mice were placed into a chamber containing isofluorane, and, when deeply sedated were perfused with 50 ml of cold phosphate buffer saline (PBS 0.1 M, pH 7.4) and brains were carefully removed(229). A camera recorded the 2 exploration sessions and measurements of the total distance (cm), the time spent on the center of the box (seconds) and velocity (cm/s) were taken.

2.4 BrdU administration

To determine the proliferation and survival of new-born cells, mice are injected with 5-Bromo-2′-deoxyuridine (BrdU, Sigma) dissolved in 0.9% saline. BrdU is a thymidine analogue which incorporates into the DNA of dividing precursor cells and thus enables to assess proliferating cells(309). Mice were intraperitoneally injected with 100 mg/kg of BrdU (Sigma Aldrich, St Louis, MO, USA) 24 h before they were killed (for proliferation analysis), or for 3 consecutive days, prior to DSS treatment (for newborn cells survival analysis)(309).

2.5 Tissue collection and Preparation

Mice were first anesthetized with isoflurane (IsoFlo®, Abbott). Approximately 1 ml of isoflurane was applied to tissue paper and placed in a closed chamber. Immediately the mice were placed in the chamber and the lid was closed for 2 min. After 2 min, mice were monitored and tested to determine whether were fully anesthetized and ready for transcardial perfusion. The respiratory rate (breathing) of fully anesthetized mice was regular and relaxed; withdrawal reflexes were absent; and there was no response to external stimuli. Then transcardial perfusion was performed. First, the heart was exposed using sharp dissecting tools and subsequently a needle of a 20 ml syringe was inserted into the left ventricle. Then, an incision was made in the right atrium to allow blood to flow out of the animal's body. The mice were perfused by applying with the syringe cold PBS at pH 7.4. When the draining blood became clear, the brain was dissected. Using appropriate dissecting tools, the head was removed and subsequently the muscle and membranous tissue were removed from the top part of the skull and the brain was extracted gently from the skull(309).

The brains were dissected from the skulls and the right hemisphere was fixed overnight in 4% paraformaldehyde for immunohistochemistry studies; the left hemisphere was dissected, and the hippocampus was isolated for protein and RNA analysis. Before being sectioned on a sliding vibratome (Leica VT1000 S), brains were cryoprotected by being immersed in 30% sucrose solution in PBS 0.1 M for 24 h at 4^0 C until they sank. Sagittal sections of 50 μm were cut in the dorsoventral axis of hippocampus (from bregma − 1.34mm to − 3.80) and stored in cryoprotective medium (30% sucrose/30% ethylene glycol in 0.1 M phosphate buffer) at − 20°C until they processed for immunofluoresence. Vibratome sections have some advantages as they allow the morphology of tissue sections to be un-disrupted due to no freezing and thawing steps and keep the antigenicity better than some other methods (e.g., paraffin embedding, ethanol fixing).

To collect the colon tissue, the ventral side of the animal was exposed and the abdomen area was wet with a 70% ethanol solution(310). Using standard dissecting scissors, the abdomen was incised by making a ventral midline incision. Then, the colon was located and transected as close to the colorectal margin as possible to free the distal colon. Carefully and slowly the whole colon was pulled down, detaching it from the surrounding mesentery. The colon was transected from the colonocecal margin to free the proximal end of the colon. Feces were removed by rinsing colon with sterile PBS by using a gavage needle attached to a 20 ml syringe(310). Tissue sampling for histological analysis and other assays was done by cutting 0.5 cm to 1.0 cm long colonic fragments (proximal, middle, or distal). In our experiments colon was harvested from all experimental mice and segments of the transverse colon (1 cm) were fixed in 4% paraformaldehyde for 2 hours on ice and then immersed in 70 % ethanol overnight before being paraffinized. Tissue samples used for measuring the colonic myeloperoxidase content were individually placed in 2 mL eppendorf tubes and frozen in liquid nitrogen and stored at -80°C.

2.6 Vibratome sectioning process

Vibratome sectioning was performed on the sliding vibratome Leica VT1000 S(311). The vibratome contains a vibrating razor blade to cut relatively thick sections of tissues. The tissues in general must be well fixed to cut easily. The following protocol was performed prior to vibratome sectioning(311):

1. The brains were first rinsed in PBS 1X at pH 7.4 and placed in a petri-dish full of PBS 1X.
2. 4 % agarose was prepared in PBS 1X. 8 g agarose was added in 200 ml PBS 1X and microwaved at 512 Hz until agarose was in solution. Solution was kept in a 55°C water bath until ready to use.
3. The right hemisphere of the brain was positioned in a sagittal plane at the bottom of a Peel-A-Way embedding mold. Any excess PBS 1X was pipped away from the mold. The mold was filled with plenty of liquid 4% agarose to cover the tissue. Agarose was set at RT within 30-40 minutes.
4. The specimen of the vibratome machine was filled with PBS 1X at RT.
5. The plastic mold was peeled away. Using a razor blade, we cut out an agarose box containing the right brain hemisphere. We ensured that the plane of section was

straight and parallel to the opposite side of the block, which constitutes the surface on which the agarose block was glued down. Since the agarose does not infiltrate the tissue, we did not trim too much agarose from around the tissue, so that the tissue was well-supported.

6. Superglue was used to attach the block to the cutting surface.
7. Sections of 50 μm thickness were taken with an adjust speed of 6 to 7 and frequency at 5.5-6 Hz. Sections were floated off the specimen in PBS and were picked up and moved to a 24 well plate filled with cold PBS 1X.
8. Sagittal sections of 50 μm were cut in the dorsoventral axis of the hippocampus (from bregma − 1.34mm to − 3.80) which consists of 50-60 sections (section thickness 50 μm).

2.7 Evaluation of colitis

2.7.1 Clinical evaluation of experimental colitis

At several time points (on days 1-7, 9-14, 16-21, 21-28, 28-35, 43-49) of the experimental colitis a disease activity score (DAS) is calculated to assess the physical status of the animals(310). The score included 3 parameters: weight loss during DSS treatment (score 0: none, score 1: 1-5 %, score 2: 6-10 %, score 3: 11-18 %, score 4: >18 %), stool consistency (score 0: normal stool, score 1: soft but formed stool, score 2: soft, score 3: very soft; wet, score 4: watery diarrhea) and presence of blood in the perianal region (score 0: negative hemocult, score 1: negative hemocult, score 2: positive hemoccult, score 3: blood traces in stool visible, score 4: gross rectal bleeding). In addition, colon length was recorded as a further index of colitis(310).

2.7.2 Assay of Myeloperoxidase (MPO) activity

1. Sample preparation for MPO analysis(310).
   i. Samples were removed from -80^0 C and placed on ice. The weight of each sample was recorded after removing any visible feces or fat by using a bent forcept. Then the colon tissue was placed into a 2 ml microcentrifuge tube. Samples were kept on ice at all times.
   ii. The appropriate amount of HTAB buffer was added according to tissue weight. If tissue weight was less than 25 mg the buffer was added at a ratio of 12.5 mg/L; if tissue weight was between 25-50 mg the buffer was added at a ratio of 25 mg/L. If tissue was greater than 50 mg, the buffer was added at a ratio of 50 mg/L.
   iii. The tissue was homogenized with a mechanical homegenizer.
   iv. The homogenized tissue was centrifuged for 6 minutes (13400 x g, 4^0 C).
   v. The supernatant was collected and the pellet was discarded. Supernatant was stored at -80^0 C.

2. MPO activity assay.
   i. o-dianisidine-dihydrochloride (o-dianisidine) solution was prepared by combining 16.7 mg of o-dianisidine dihydrochloride, 90 mL of dH2O, and 10 mL of potassium phosphate buffer. This solution was prepared fresh for every assay.
   ii. Tissue homogenate (7 μl) was added in triplicate into a 96 well-plate.

iii. 50 µl of diluted H_2O_2 (4 µl of 30% H_2O_2 in 96 µl of dH_2O) was added to the o-dianisidine mixture.

iv. 200 µL of the o-dianisidine mixture containing H_2O_2 was added to each of the wells using multi-channel pipette.

v. Absorbance was measured at 450 nm using a spectrophotometer and three readings were taken at 30 second intervals.

vi. MPO activity calculation. MPO activity was measured in units (U) of MPO/mg tissue, where one unit of MPO was defined as the amount needed to degrade 1 µmoL of H_2O_2 per minute at room temperature. Considering that one unit (U) of MPO= 1 µmoL of H_2O_2 split and that 1 µmoL of H_2O_2 gives a change of absorbance of 1.13 x 10-2 nm/min, units of MPO in each sample were determined as change in absorbance [ΔA(t2-t1)]/Δmin x (1.13 x 10-2). To get units per mg of tissue we used calculated the tissue: buffer ratio. For example, if a tissue: buffer ratio of 50 mg/mL was used, in 7 µL of homogenate, there was 0.35 mg of tissue. Therefore, to get units per mg tissue, we divided the units of MPO by 0.35. A sample calculation using absorbance values (nm) is included in the below table (assuming that the sample has been added in triplicate):

Sample	Time 0 sec			Time 30 sec			Time 60 sec		
	1	2	3	1	2	3	1	2	3
	0.052	0.053	0.055	0.061	0.062	0.064	0.081	0.083	0.085

Average at Time 0 sec= (0.052 + 0.053 + 0.055)/3= 0.053 nm

Average at Time 30 sec= 0.062 nm

Average at Time 60 sec= 0.083 nm

Change in absorbance (ΔA) from 0 to 30 sec/ mg tissue (assuming 50 mg/mL of tissue: buffer ratio) = $[(0.062-0.053)/(1.13 \times 10^{-2})]/0.35= 2.257$

Change in absorbance (ΔA) from 30 to 60 sec/ mg tissue= 5.3

*MPO activity (U/mg tissue) = average of ΔA (0-30) and ΔA (30-60) = 3.778.

2.7. 3 Qualitative Histological analysis (H&E staining)

To evaluate histological damage of colitis severity a small fragment (0.5-2 cm) of the colon was cut and placed in a tissue cassette and submerged in buffered 4% PFA solution(310). Paraffin embedded colon was sectioned in a microtome and stained with hematoxylin/eoxin (H&E) using the following protocol(310):

1. The paraffin sections (5 µm) were dried for 5-10 minutes in a metal plate at 60^0 C.
2. The slides with paraffin sections were placed in a slide holder (glass or metal).
3. Deparaffinization and rehydration of paraffin sections:
    i. 1 x 5 minutes and 1x 10 minutes xylene (blot excess xylene before going into ethanol).

ii. 3 x 3 minutes 100 % ethanol.
iii. 1 x 1 minute 95 % ethanol.
iv. 1 x 1 minute 75 % ethanol.
v. 1 x 1 minute 50 % ethanol.
vi. Tap water 5 minutes.

While sections in water the hematoxylin was filtered. The excess water was blotted before going to hematoxylin.

4. Hematoxylin staining for 1 minute.
5. Then, we rinsed with tap water 3 times. The excess water was blotted before going to eosin.
6. Eosin staining for 1 minute.
7. We rinsed with tap water 3 times.
8. 30 seconds 80 % ethanol.
9. 30 seconds 90 % ethanol.
10. 2x 1 minute 100 % ethanol.
11. 2x 5 minutes xylene.

The slides were coversliped using Permount (xylene based). One drop of Permount was placed on the slide using a Pasteur pipette taking care to leave no bubbles. The coverslip was angled and let fall gently onto the slide. The Permount was spread beneath the coverslip, covering all the tissue. The slides were dried overnight in the hood.

Colon fragments were taken from the proximal, mid-colon, or distal section of the colon. Images of H&E stained sections are taken by a brightfield microscope (Leica DM LS2) using 10x and 40x objective. The presence of areas with strong transmural inflammation with loss of crypt structure and depletion of goblet cells, as well as the presence of ulceration, crypt abscesses and neurotrophilic infiltrates were evaluated as signs of DSS induced colitis(310).

2.8 Immunohistochemistry

Immunostaining was performed in 1 to 6 or 1 to 12 series of 50 μm free floating sagittal sections (300 μm or 600 μm apart, respectively)(312,313).

For BrdU immunostaining the followed protocol was used(309):

1. Free floating sections were washed in PBS 1X at pH 7.4 on a shaker. The sections were placed on a 24 well plate.
2. Denaturation of DNA by incubation of sections in 2 M HCl for 20 minutes on a hybridizer oven at 40^0 C. The HCl solution was pre-heated for at least 1 hour. Denaturation of DNA allows access for the anti-BrdU antibody so incomplete denaturation causes problems.
3. Neutralization of the HCl by incubating the sections in Sodium Borate 0.1 M pH 8 at RT on a shaker twice for 15 minutes.
4. The sections were rinsed three times, 5 min each in PBS 1X (at pH 7.4).

5. Incubation of sections with blocking solution consisting of 4% NDS, 0,3 % Triton X-100, PBS 1 X at pH 7.4 for 60 minutes on a shaker.
6. The sections were incubated with primary antibodies (BrdU, NeuN, GFAP and Sox2) in blocking solution overnight at 4^0 C on a shaker (Table 1).
7. The sections were rinsed three times, 5 min each in PBS 1X (at pH 7.4).
8. Then, the sections were incubated with fluorochrome-conjugated secondary antibody in the dark, diluted in 4 % NDS, PBS 1X (at pH 7.4) with 0.3% Triton X solution (detergent such as Triton-X can be added to the secondary antibody diluent to reduce hydrophobic interactions between tissue and reagent proteins, thus reducing non-specific binding of secondary antibodies) for 2 hours at RT on a shaker (Table 2).
9. The sections were rinsed three times, 5 min each in PBS 1X (at pH 7.4) in the dark.
10. A coplin jar was filled with PBS 1X and each section was transferred there.
11. The sections carefully transferred to slides with a soft brush.
12. Mounting medium was added (DAPI with Vectashield) and coverslips were placed.
13. Mounting sections can be kept at 4^0 C for up to 6 months before imaging with a microscope.

Denaturation of DNA is often detrimental to other antigens, particularly surface antigens and receptors. In our experimental setup the DCX antigen was affected by HCl treatment(309). For this reason, we performed antigen retrieval treatment on a hybridizer oven or on a microwave.

Antigen retrieval protocol*:

1. Free floating sections were washed in PBS 1X at pH 7.4 on a shaker. The sections were placed on a 24 well plate.
2. The sections were transferred to 10 mM sodium citrate buffer (at pH 6), preheated to 80^0 C in a hybridizer oven or in a beaker containing 50 ml 10 mM sodium citrate buffer (at pH 6.0)
3. The sections were kept in 10 mM sodium citrate buffer (at pH 6) at 80^0 C for 30 min or for 2-2.5 minutes in a microwave at 512 Hz.
4. Then, sections were kept in 10 mM sodium citrate buffer (at pH 6) while allowing the sections to cool to room temperature.
5. The sections were rinsed three times, 5 min each in 0.1 M PBS (at pH 7.4).
6. The sections were incubated with blocking solution consisting of 4% NDS, 0,3 % Triton X-100, PBS 1 X at pH 7.4 for 60 minutes on a shaker.
7. The sections were incubated with primary antibodies (Ki67, DCX, Caspase3, Iba-1, CD31 and Arc) in blocking solution overnight at 4^0 C on a shaker (Table 1).
8. The sections were rinsed three times, 5 min each in PBS 1X (at pH 7.4).
9. Sections were then incubated with fluorochrome-conjugated secondary antibody in the dark, diluted in 4 % NDS, PBS 1X (at pH 7.4) with 0.3% Triton X solution for 2 hours at RT on a shaker (Table 2).
10. Sections were rinsed three times, 5 min each in PBS 1X (at pH 7.4) in the dark.

11. A coplin jar was filled with PBS 1X and each section was transferred there.
12. Each section was transferred carefully to slides with a soft brush.
13. Mounting medium was added (DAPI with Vectashield) and coverslips were placed.
14. Mounting sections can be kept at 4^0 C for up to 6 months before imaging with a microscope.

*The antigen retrieval protocol can be used to stain BrdU. For triple staining with BrdU, DCX and NeuN or for double staining with BrdU, Ki67 or Arc the above protocol is performed (27).

Table 1. Primary antibodies

Antibody	Dilution	Antigen specificity	Protocol	Source, catalog no.	Monoclonal or Polyclonal, host species
BrdU	1:200	This antibody reacts with BrdU (5-Bromo-2'-deoxyuridine) in single stranded DNA, BrdU attached to a protein carrier or free BrdU. It detects nucleated cells that have had BrdU incorporated into their DNA during S phase.	HCl treatment or Antigen retrieval treatment with microwave	Abcam, ab6326, BU1/75 (ICR1)	Rat monoclonal
Sox2	1:200	Sox2 Antibody detects endogenous levels of total Sox2 protein.	HCl treatment	Cell signaling, #2748	Rabbit polyclonal
DCX	1:100	raised against amino acids 123-402 mapping at the C-terminus of Doublecortin of human origin epitope mapping at the C-terminus of Doublecortin of human origin	Antigen retrieval treatment either with hybridizer oven /microwave	Santa Cruz (E-6): sc-271390 or Santa Cruz (C-18): sc-8066	Mouse monoclonal, Goat polyclonal
NeuN	1:500	NeuN (D3S3I) Rabbit mAb recognizes	HCl treatment or Antigen retrieval	Cell Signaling, (D3S3I)	Rabbit Polyclonal

		endogenous levels of total NeuN protein.	treatment with microwave	#12943	
GFAP	1:500	Enriched bovine glial filaments	HCl treatment	ThermoFisher Scientific # 13-0300	Mouse monoclonal
Iba-1	1:600	raised against a synthetic peptide corresponding to the Iba1 carboxy-terminal sequence, which is conserved among human, rat and mouse Iba1 protein sequences.	Antigen retrieval treatment with hybridizer oven	Wako .# 019-19741	Rabbit Polyclonal
Caspase 3	1:100	Caspase-3 Antibody detects endogenous levels of full length caspase-3 (35 kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa).	Antigen retrieval treatment with microwave	Cell signaling, #9662	Rabbit Polyclonal
Ki67	1:200	Synthetic peptide conjugated to KLH derived from within residues 1200 - 1300 of Human Ki67.	Antigen retrieval treatment either with hybridizer oven /microwave	Abcam ab15580	Rabbit Polyclonal
CD31	1:100	129/Sv mouse-derived endothelioma cell line tEnd.1	Antigen retrieval treatment either with hybridizer oven /microwave	BD Bioscience 550274	Rat polyclonal
Arc	1:200	Recombinant protein corresponding to AA 1 to 396 from mouse Arc (UniProt Id: Q9WV31)	Antigen retrieval treatment either with hybridizer oven/microwave	Synaptic Systems 156-004	Guinea Pig polyclonal

Table 2. Secondary Antibodies-Conjugated with Fluorochromes

Antibody	Antigen Specificity, Species reactivity	Conjugate	Dilution	Source, Catalog. no.	Polyclonal or monoclonal, host species
IgG (H+L)	Gamma Immunoglobin, Rabbit	Alexa Fluor® 488	1:500-1:600	Invitrogen, ThermoFisher Scientific # R37118	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Rabbit	Alexa Fluor® 568	1:500-1:600	Invitrogen, ThermoFisher Scientific # A10042	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Rabbit	Alexa Fluor® 647	1:500-1:600	Invitrogen, ThermoFisher Scientific # A-31573	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Rabbit	Alexa Fluor® 633	1:500-1:600	Invitrogen, ThermoFisher Scientific # A-21070	Polyclonal Goat / IgG
IgG (H+L)	Gamma Immunoglobin, Mouse	Alexa Fluor® 488	1:500-1:600	Invitrogen, ThermoFisher Scientific # R37114	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Mouse	Alexa Fluor® 568	1:500-1:600	Invitrogen, ThermoFisher Scientific # A10037	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Rat	Alexa Fluor® 488	1:500-1:600	Invitrogen, ThermoFisher Scientific # A-21208	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Rat	Alexa Fluor® 568	1:600	Abcam, ab175475	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Goat	Alexa Fluor® 488	1:500-1:600	Invitrogen, ThermoFisher Scientific # A-11055	Polyclonal Donkey / IgG
IgG (H+L)	Gamma Immunoglobin, Guinea Pig	Alexa Fluor® 488	1:500-1:600	Invitrogen, ThermoFisher Scientific # A-11073	Polyclonal Goat / IgG

2.9 Image analysis and quantification

Stereological analysis of the number of the cells was performed with confocal microscopy (Leica TCS SP5 with AOBS) one in six series or one in 12 series of 50 μm free floating sagittal sections (300 μm or 600 μm apart, respectively)(312–314). The total estimated number of cells within the dentate gyrus, positive for each of indicated markers, was obtained multiplying the average number of positive cells per section by the total number of 50- μm sections comprising the entire dentate gyrus (about 50-60 sections), as described(312). Confocal image z-stacks were captured through the entire slice thickness at 1.5 μm (20x lens) or 0.84 μm (63x lens) optical steps and used for double/triple-labelled cell counts.

Quantification of Type 1, Type 2a, Type 2b and Type 3 cells. Type 1 cells were identified as $BrdU^+/GFAP^+/Sox2^+$ with a radial glial like morphology, and Type 2a cells as $BrdU^+/Sox2^+/GFAP^-$ with a non-radial glial like morphology. To quantify Type 1 and Type 2a cells, we have performed triple immunohistochemical labelling using the BrdU, GFAP and Sox2 markers. Furthermore, cells that express Sox2 and the marker for the immature neurons doublecortin (DCX) were identified as Type 2b cells ($BrdU^+/Sox2^+/DCX^+$). Type 3 cells were identified as $BrdU^+/Sox2^-/DCX^+$ and were considered as proliferating immature neurons. To quantify Type 2b and Type 3 cells we performed triple immunohistochemical staining using the BrdU, Sox2 and DCX markers. Quantification was performed manually on ImageJ software on confocal images and was confirmed on single optical sections, for 30-60 cells per animal. The ratio of neuronal phenotypes was multiplied by the total number of BrdU-positive cells and yielded an estimation of the absolute numbers within the sub-granular and granular cell layers(313).

Quantification of Immature and Mature newborn neurons. Immature neurons were identified as $BrdU^+/DCX^+/NeuN^+$ while mature newborn neurons as $BrdU^+/DCX^-/NeuN^+$ and counted in the granule cell layer(293).

Quantification of Arc-expressing cells. The density of Arc+ cells was relatively low (around 2.2–3.4% of mature granule cells expressed Arc following behavioral testing) and therefore it was possible to quantify the number of Arc+ cells using a confocal microscope in the entire DG(162).

Quantification of $BrdU^+/NeuN^+/Arc^+$ cells. The fraction of $BrdU^+$ cells co-expressing $BrdU^+/NeuN^+/Arc^+$ among all $BrdU^+$ cells was calculated by manual cell counting on ImageJ software on confocal images and was confirmed on single optical sections, for 30 cells per animal(162).

Quantification of $NeuN^+$ cells in the dentate gyrus. To estimate the total number of mature neurons in the dentate gyrus, sections were stained for the mature neuronal marker, NeuN. Using a method adapted from a previous report, two z-stacks per dentate gyrus (one each from the upper and lower blades) were collected from six mice at 20x magnification using the confocal microscope. The total number of $NeuN^+$ cells in each 50-μm stack was counted manually and the total area of the middle plane was measured. From this we calculated the

density of $NeuN^+$ cells in the dentate gyrus(162). From our analysis of Arc^+ cells, we then determined the proportion of mature neurons expressing Arc following behavioral testing.

Quantification of Ki67, Sox2, DCX, Iba-1 and GFAP. Cells expressing Ki67, Sox2, and DCX were counted in the sub-granular and granule cell layers, whereas cells expressing Iba1 and GFAP were counted in the whole DG. All numbers are based on the numbers of individual cell nuclei(314). Morphological criteria were applied to distinguish the horizontal astrocytes from radial glia-like cells(104). Briefly, horizontal astrocytes have the characteristic astrocytic features (star-shaped) and are localized mainly in the hilus and Molecular layer of the DG, whereas radial glia-like cells reside only in the SGZ and GCL layer of the DG(315).

2.10 Sholl analysis of microglia cells

Z-stack confocal images of microglia cells in the region of the Dentate Gyrus were acquired at 1.5 μm interval using a 20x objective. Consecutive Z-stack Images were converted to a maximum intensity projection image and subsequently converted to a binary image by thresholding to include microglial processes using Image J software(316). From each mouse we isolated randomly 5 to 10 microglia cells per DG. Using the line segment tool, we draw a single line from the center of the microglia soma to its longest process. Next, using the Sholl analysis plugin, we defined the first shell to be 10 μm outside of the cell body (to exclude the soma from Sholl analysis) and set each step to be 5 μm and finally selected the linear profile to be concluded in the analysis. Sholl analysis was manually performed for each cell by counting the number of intersections between microglia branches and each increasing circle to create a Sholl plot(316). Then, from these data we determined the maximum branch length (μm, the maximum radius at which a branch intersection occurred), the number of primary branches (Np, the number of branches that originated from the microglia soma), the process maximum (Nm, the maximum number of intersections for the cell) and the critical value (Cr, the distance from the soma where Nm occurred)(316). From these parameters, a Shoenen ramification index was calculated (Nm/Np) to quantify cell branching density (316). In addition, we analyzed manually the cell soma area of each microglia cell using ImageJ software.

2.11 Immunobloting

2.11.1 Protein extraction

1. The hippocampus was dissected with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. The tissue was placed in round-bottom microfuge tubes or Eppendorf tubes and immerse in liquid nitrogen to “snap freeze”. Samples were stored at -80°C for later use or on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, ~300 μl lysis buffer* was added rapidly to the tube and homogenization was performed with an electric homogenizer. The blade was rinsed twice with another 2x300 μl lysis buffer. Then the samples were maintained in constant agitation for 2 hours at 4°C (e.g on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present (protein extract should not be too diluted to avoid loss of protein and large volumes of

samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml, optimal concentration is 1-5 mg/ml).

4. The homogenates were centrifuged for 20 min at 12000 rpm at 4°C in a microcentrifuge. The tubes were removed gently from the centrifuge and placed on ice and the supernatant was aspirated and placed in a fresh tube kept on ice; the pellet was discarded.

***Cell/Tissue extraction buffer recipe**

1) 100 mM Tris, pH 7.4

2) 150 mM NaCl

3) 1 mM EDTA

4) 1 mM EGTA

5) 1% Triton X-100

6) 0,5 % sodium deoxycholate

Additional Reagents

Phosphatase and Protease inhibitors (1:100, ThermoFisher).

2.11.2 Determination of protein concentration- Bradford Assay

In 2 ml tubes 1500 μl Bradford Reagent (Bio-rad) and 50 μl sample (25 μl sample+ 25 μl ddH2O) were placed. As blank Bradford Reagent + 50 μl ddH2O was used. Measurements were taken in NanoDrop at 595 nm. Then, a standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in μg/ml (x-axis). The unknown sample concentration was determined using the standard curve. If the samples were diluted, the final concentration were adjusted of the unknown samples by multiplying by the dilution factor used.

2.11.3 Sample preparation

The denaturation of samples was performed by heating at 80^0 C for 10 minutes using a loading buffer with the anionic denaturing detergent sodium dodecyl sulfate (SDS). The standard loading buffer was called 2X Laemmli buffer. The 2X was mixed in a 1:1 ratio with the sample.

Laemmli 2X buffer

4% SDS

10% 2-mercaptoehtanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris HCl

The pH should be at 6.8.

It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size by using ß-mercaptoethanol or dithiothreitol (DTT). During protein sample treatment the sample was mixed by vortexing before and after the heating step for best resolution.

2.11.4 Electrophoresis

Polyacrylamide Gels.

Polyacrylamide gels were formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization was initiated by the addition of ammonium persulfate along with TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups. According to the size of the protein of interest the gels are made according to the table below. The smaller the size of the protein of interest, the higher the percentage of mono/bis. The bigger the size of the protein of interest, the lower the percentage of mono/bis.

Table 3. Polyacrylamide Gels

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

2.11. 5 Sample Loading and running the gel

The samples were loaded into the wells with a 20 µl pipet using gel loading tips. 18-20 µg of protein was loaded. As molecular weight marker we use the PiNK Plus Prestained Protein Ladder (GeneDirex, Inc.).

Then the gels were submerged in migration buffer which normally contains SDS. A standard migration buffer (also called running buffer) for PAGE is 1X Tris-glycine: 25 mM Tris base, 190 mM glycine, 0.1% SDS (the pH should be around 8.3). In our experimental setup we used the low voltage method for running the gels. 40 V for 10 min and then 100 V until the dye front reaches the bottom (expected run time 80-90 minutes).

2.11.6 Transfer of proteins

Transfer was done in wet conditions. In wet transfer, the gel and membrane are sandwiched between sponge and paper (sponge/paper/gel/membrane/paper/sponge) and all are clamped tightly together after ensuring no air bubbles have formed between the gel and membrane. The sandwich was submerged in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but the

membrane stops them, binds them, and prevents them from continuing on. The standard buffer for wet transfer is the same as the 1X Tris-glycine buffer used for the migration/running buffer without SDS but with the addition of methanol to a final concentration of 20%. For proteins larger than 80 kD, it is recommended that SDS is included at a final concentration of 0.1%. In our experimental setup we used the nitrocellulose membrane. Before transferring we incubated the membrane in ice cold transfer buffer for 5-10 minutes. Also, the gel needs to equilibrate for 3-5 minutes on ice cold transfer buffer. Power conditions in transfer: 300 mA for 1:30 hour on ice.

2.11.7 Visualization of proteins in membranes: Ponceau Red

To check for success of transfer, the membrane was washed in TBST (for a TBST recipe, see below). The stock Ponceau Red was diluted 1:10. The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid. We incubated on an agitator for 5 min and then washed extensively in water until the water was clear and the protein bands were well-defined. The membrane was destained completely by repeated washing in TBST or water.

TBS 10x (concentrated TBS)

24.23 g Trizma HCl

80.06 g NaCl

Mix in 800 ml ultra pure water.

pH to 7.6 with pure HCl.

Top up to 1 L.

TBST

For 1 L: 100 ml of TBS 10x + 900 ml ultra pure water + 1ml Tween 20©

2.11.8 Blocking the membrane

The membrane was blocked with non-fat milk or BSA to prevent non-specific background binding of the primary and/or secondary antibodies to the membrane. Milk is commonly used as a blocking solution for the membranes but is not recommended for studies of phospho-proteins (milk contains casein which is a phospho-protein; it causes high background because the phospho-specific antibody detects the casein present in the milk).

To prepare a 5% milk or BSA solution, we weighted 5 g per 100 ml of Tris Buffer Saline Tween20 (TBST) buffer. We mixed well and filtered. Failure to filter can lead to “spotting” where tiny dark grains will contaminate the blot during development. Incubate for 1 hour at RT under agitation. We rinsed for 5 seconds in TBST after the incubation.

2.11.9 Incubation with the primary antibody

The primary antibody was diluted in TBST buffer at the suggested dilution of the datasheet and incubated overnight at 4^0 C under agitation. The primary antibodies used in this study are depicted in the below table.

Table 4. Primary antibodies for Western blotting

Antibody	Dilution	Source, catalog no.	Monoclonal or Polyclonal, host species
NFkB-p65	1:1000	Cell signalling, 8242	Rabbit polyclonal
mTOR	1:1000	Cell signalling, 2972	Rabbit polyclonal
p-mTOR	1:1000	Cell signalling, 2971	Rabbit polyclonal
PI3K p85	1:100	Santa Cruz, sc-423	Rabbit polyclonal
GSK3β	1:2000	Santa Cruz, sc-9166	Rabbit polyclonal
^{pSer9}GSK3β	1:1000	Cell signalling, 9336	Rabbit polyclonal
GFAP	1:1000	ThermoFisher Scientific, 13-0300	Mouse monoclonal
GAPDH	1:10000	Cell signalling, 2118	Rabbit polyclonal
Actin	1:10000	Cell signalling, 4970	Rabbit polyclonal

2.11.10 Incubation with secondary antibody

The membrane was washed in TBST 3x10 minutes to remove residual primary antibody. Then the HRP-conjugated antibodies were diluted in blocking buffer at a range of dilutions (1:2500-1:10000). The membrane was incubated with the secondary antibody for 2 hours on constant agitation at RT.

Table 5. Secondary antibodies-HRP conjugated

Secondary conjugated with HRP	Dilution	Source, catalog no.
IgG, HRP conjugate, goat anti-rabbit	1:5000-1:10000	Millipore, 12438
IgG, HRP conjugate, goat anti-mouse	1:2500-1:5000	Millipore, 12439

2.11.11 Developmental methods

Chemiluminescence signals were revealed with Immobilon Western Chemiluminescent HRP Substrate (Millipore). Membranes were immediately read in a Kodak Image Station 4000 mm PRO, and protein levels were quantified using Image Studio Lite Ver 5.2.

2.11.12. Stripping and reprobing

Stripping is the removal of primary and secondary antibodies from a Western blot membrane. For the determination of phospho- protein levels in hippocampal lysates, membranes were first probed with the phospho-specific antibody, stripped and re-probed with a second antibody for the total protein. In our experimental setup we used the mild stripping procedure as the harsh stripping destroyed the antigens of the proteins.

Mild stripping

Buffer, 1 L

15 g glycine

1 g SDS

10 ml Tween20

Adjust pH to 2.2

Bring volume up to 1 L with ultrapure water.

Membrane incubation

We used a volume that cover the membrane. We incubated at room temperature for 5-10 minutes.

The buffer was discarded.

5-10 minutes fresh stripping buffer.

The buffer was discarded.

10 minutes PBS 1X

10 minutes PBS 1X

5 minutes TBST

5 minutes TBST

Ready for blocking stage and reprobed with the primary antibody.

2.12 Cytokine analysis (IL-1β, IL-6, TNF-a and IL-10) by ELISA

2.12.1 Sample preparation

Serum samples: Blood was collected by cardiac puncture in Eppendorf tubes and was allowed to clot for 2 hours at room temperature. Then samples were spined for 8 minutes at 8,000 rpm at 4^0 C and the supernatant (serum) was collected(317).

Tissue samples: Freshly harvested hippocampus was immediately frozen in liquid nitrogen and stored at -80^0 C.

Protein extraction:

1) The samples were weighted.
2) Samples were thawed on ice.
3) Samples were placed in a concentration of 50 mg of tissue per 1ml of sterile HBSS containing protease inhibitors (Sigma-Aldrich).
4) Samples were homogenized using a T-8 homogenizer (IKA-WERKE).
   The homogenates were centrifuged at 400g for 15 minutes at 4^0 C and the supernatants were collected and stored at -80^0 C.

2.12.2 ELISA protocol

1) Plates were coated with 100 µl of capture antibodies at 1 µg/ml in coating buffer. Then, we incubated for 3 hours at RT or overnight at 4 °C.

2) The coating antibodies were dumped and 200 µl of blocking solution were added to block free binding sites on the plate. Incubation for 1 h (minimally) at RT.
3) Then, we washed for 4 times by flicking off the plated by adding 250 µl per well of wash buffer each time. Commercially available ELISA plate washers work well for this purpose. However, washing can also be carried out by submerging the plates one by one in a container with wash buffer, by using a squirt bottle, or by manual pipetting.
4) After the final wash, we dried the plates on a piece of paper towel.
5) Then, we prepared the dilutions of standard samples and samples in PBSB.
6) We added 100 µl per well of samples (serum and hippocampal homogenates) and standards. We set up duplicate wells for each sample or standard. We also included negative samples containing only PBSB as blank.
7) Incubation overnight at 4 °C.
8) Washing four times by flicking off the plate by adding 200 µl per well of wash buffer each time. After the final wash, the plates were dried on a piece of paper towel.
9) After the final wash, we added 100 µl per well of biotinylated detection antibodies diluted in PBSB to a concentration of 1 µg/ml.
10) Incubation for 1–2 h at room temperature.
11) Washing four times by flicking off the plate by adding 200 µl per well of wash buffer each time. After the final wash, the plates were dried on a piece of paper towel.
9) After the final wash, we added 100 µl per well of biotinylated detection antibodies.
12) 100 µl per well of Avidin-HRP was added, diluted 2,000-fold in PBSB.
13) Incubation 30 min at room temperature.
14) Washing four times by flicking off the plate by adding 200 µl per well of wash buffer each time. After the final wash, the plates were dried on a piece of paper towel.
15) Then, 100 µl of TMB substrate solution per well was added. Signal will show up as a blue color, which turns yellow after addition of Stop solution. Importantly, the Stop solution should be added in the same order as the substrate solution at the same pace, to avoid false differences.
16) Absorbance was measured at 450 nm and was stable for up to 1 h after addition of Stop solution.

2.12.3 Data analysis

In general, the first analysis step was to make a standard curve using the serial dilutions of protein standards(317). This analysis models protein concentration as a function of the OD. Since the standard curve tends to be sigmoid, linear regression is not recommended. Point-to-Point, Cubic Spline, or four Parameter are the most accurate curve-fitting routines. Linear regression is used to include the portion of the standard curve that falls within the linear range(317). Before proceeding to the analysis of the unknown samples, we made sure that the standard curve is reliable, as judged by an R^2 value greater than 0.98. Outliers are frequently found at higher concentrations, where saturation has occurred. These should be discarded. If one of a duplicate standard obviously falls outside the standard curve, it is also best to discard that sample and recalculate the curve. Subsequently, we ensured that experimental samples fall within the range of the assay.

2.13 Flow-cytometry analyses

2.13.1 Tissue processing for hippocampal microglia isolation

All procedures were carried out on ice. After removal of the meninges, hippocampus was rapidly dissected and placed in a Petri dish containing 2 ml of ice cold PBS. Identical regions from 4 mice were pooled per experiment to increase microglia yield. Hippocampi were minced with a razor blade and transferred to a 70 μm pore size strainer (BD Biosciences) on top of a 50 ml canonical tube (Greiner Bio-One). Tissue was then gently dissociated and mashed through the strainer to reach a single cell suspension. An additional 30 ml of PBS was added to the tube containing the cell suspension, and then the tubes were centrifuged for 10 minutes at 300x *g* at 4^0 C(318).

2.13.2 Density Gradient Centrifugation

The supernatant was discarded and the remaining cell pellet was resuspended in 1 ml 40 % Percoll and transferred to a new 15 ml polysterene tube after witch an additional 7 ml 40 % Percoll was added. Then 4 ml of 80 % Percoll was gently layered underneath the 40 % Percoll layer and subsequently 3 ml PBS/FCS/EDTA buffer was layered on the top of 40 % Percoll using a Pasteur pipette. The density gradient was centrifuged at 600x *g* at 4^0 C for 30 minutes with minimum deceleration(318).

2.13.3 Microglia collection

Two distinct layers were apparent after centrifugation. The top layer between the PBS/FCS/EDTA and 40 % Percoll gradient consisted mainly of thick, viscous myelin. The lower layer at the interphase between the 40 and 80 % Percoll phase appeared quite faint and contains highly enriched microglia. First, the top layer was carefully removed and then, using a new Pasteur pipette, the microglia containing interphase was aspirated and transferred to a 15 ml polysterene tube. Cells were washed twice with 14 ml of PBS/FCS/EDTA buffer and after adding another 14 ml PBS/FCS/EDTA buffer, the cells were centrifuged for 7 minutes at 300x *g* at 4^0 C and the buffer was discarded(318).

2.13.4 Fluorescence-Activated Cell Sorting (FACS)-Staining of Microglia cells

Immediately after Percoll gradient separation and washing steps, cells were stained with the following antibodies: mouse CD45-FITC (Clone:30-F11) (1:100), CD45-Pacific Blue (Clone:30-F11) (1:100), CD11b-PE-Cyanine 5 (Clone:M1/70) (1:100), Ly6C-PE-Cyanine 7 (Clone:HK1.4) (1:100), nitric oxide synthase 2 (NOS-2)-PE Cyanine 7 (Clone: CXNFT) (1:100) (eBioscience), Arginase-1 (Arg-1)-FITC (R&D Systems) (1:100) for 30 minutes at 4^0 C diluted in PBS and shielded from light. For intracellular staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (Becton Dickinson). Subsequently, cells were rinsed twice in PBS, pelleted at 1200 rpm for 5 minutes at 4^0 C and resuspended in 300 μl PBS before they were filtered over a 70 μm size strainer to obtain single cell suspension ready for FACS analysis and sorting. FACS acquisition was performed with the cytometer BD FACSAria II (Becton Dickinson) and the data were analyzed using the FlowJo software 8.7 (Tree Star, Inc., Ashland, OR).

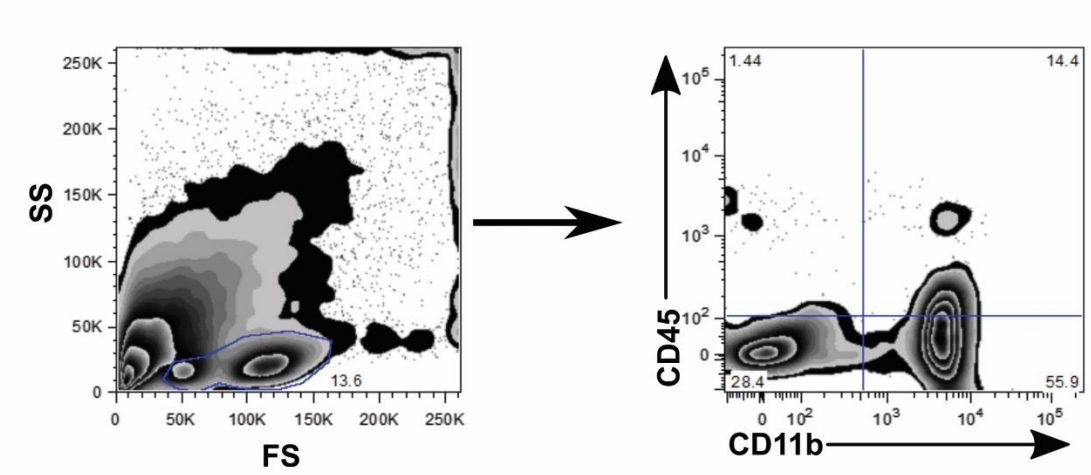

Figure 1. *Gating strategy followed to distinguish microglia vs infiltrating peripheral macrophages based on the expression levels of CD45 and CD11b: microglia being CD45low/-/CD11b+ and macrophages being CD45high/+ /CD11b+. FS, Forward scatter; SS, side scatter.*

2.14 Quantitative real-time RT-PCR

Total RNA was isolated from hippocampus or colon using TRI reagent (Millipore Sigma).

2.14.1 RNA isolation

1. Snap-freeze the tissue to be extracted. Powder on dry ice or using a precooled mortar and pestle, grind the frozen tissue under liquid nitrogen.
2. 1 ml of TRizol was added per 50-100 mg of tissue.
3. The samples were solubilized for 5 minutes at RT.
4. Chloroform was added in a concentration of 0.2 ml per ml of TRIzol and then vortexed for 15 seconds. The mixture was allowed to stand for 2-3 minutes at RT.
5. Centrifuge at maximum speed in a microcentrifuge or at 10,000g in a centrifuge for 10 min at RT. This produced two phases: an upper clear one containing RNA and a lower red one that contains protein. DNA is at the interface.
6. The upper clear phase was transferred to a fresh tube, taking care not to disturb the interface.
7. Optional. If the expected RNA concentration is ≤10 µg/mL, a carrier was added (e.g., glycogen or GlycoBlue) to the upper phase before proceeding to Step 7.
8. 0.5 mL of isopropanol per each milliliter of the clear phase was added. Then, we mixed vigorously by rapid shaking or vortexing and let stand for 10 min.
9. The precipitated RNA was collected by centrifugation at maximum speed in a microcentrifuge or at 10,000g in a centrifuge for 10 min at 4°C.
10. The supernatant was carefully decanted and any remaining liquid was removed by a Pasteur pipette. The white RNA pellet appeared as a triangle extending from the bottom of the tube slightly upward.
11. The RNA pellet was washed once with 75% ethanol: 75% ethanol 1 ml/ml TRIzol.
12. Then, we vortexed and centrifuged for 5 min at 4^0 C by less than 8000 rpm.

13. Air dry RNA pellet (5-10 min). The RNA pellet was not let to dry completely as this would greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. RNA was dissolved in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.
14. RNA was dissolved in 0.03–0.06 ml RNase-free water or 0.5% SDS solution or in DEPC-ddH_2O.
15. Mixed well with pipette.
16. RNA was incubated for 10 min at 55-60^0 C and stored at -80^0 C.

2.14.2 Removal of genomic DNA from RNA preparations

DNase I is an endonuclease that digests single- and double-stranded DNA. It hydrolyzes phosphodiester bonds producing mono- and oligodeoxyribonucleotides with 5'-phosphate and 3'-OH groups.

Method for removal of genomic DNA.

1. In an RNase free tube we added:
   i. 1 µg RNA
   ii. 10x reaction buffer with $MgCl_2$ 1 µl
   iii. DNase I, RNase- free 1 µl (1U)
   iv. DEPC-treated water up to 10 µl
2. Incubation at 37^0 C for 30 minutes.
3. Then, we added 1 µL 50 mM EDTA and incubate at 65°C for 10 min. RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent. Alternatively, we used phenol/chloroform extraction.
4. The prepared RNA was used as a template for reverse transcriptase.

2.14.3 Determining the Yield and Quality of Purified RNA using a Nanospectrophotometer

The easiest way to determine the quantity of RNA in a sample is to measure the absorbance at 260 nm (A260) using a spectrophotometer. Because the bases in RNA absorb ultraviolet (UV) light in the 250- to 265-nm range, one can use this property to quantitatively measure the concentration of an RNA solution, using an average absorbance for the four nucleotide bases. The advent of nanospectrophotometers, such as the Thermo-Fisher NanoDrop and GE Healthcare NanoVue, has greatly increased the ease, sensitivity, and accuracy of determining the concentration of low-volume (microliters) samples by UV absorbance.

Procedure for measuring RNA in nanophotometer. We placed 1.0-1.5 µL of the RNA sample onto the sample pedestal. The UV absorbance of the sample was then read either at a fixed wavelength or in a UV-visible scan(319). Typically, pure RNA (or DNA) samples are read at A_{260} and A_{280}. When adding a sample to the pedestal, we ensured that the sample was placed over the "eye" (the eye is the little metal circle with the tiny hole in the NanoDrop). Bubbles are incompatible with accurate readings. Although the instructions may claim that only 1 µL is needed, in practice a larger volume (1.3-1.5 µL) produce more reliable readings because the droplet have better optical characteristics. Small-volume

droplets can give incomplete coverage across the pedestal. It is possible to saturate the spectrophotometer with high-concentration solutions of RNA or DNA. This might cause an underestimate of the true concentration(319). We tried to obtain readings using solutions at ≤2.5 μg/μL. For samples that give higher readings (>2.5 μg/μL), we diluted at 1:10 and read the dilution to get the most accurate reading.

Although measuring A_{260} is generally a reliable way to quantitate RNA concentrations, this method can be confounded if the RNA is contaminated with DNA, protein, or phenol, all of which absorb some UV light at 260 nm. A diagnostic for protein contamination is absorbance at 280 nm(319). Phenol and TRIzol Reagent both absorb at 270 nm and 230 nm. For purified RNA the ratio A_{260}:A_{280} should be ~2.0 because the unpaired bases in RNA absorb more UV light than the base-paired bases in duplex DNA. If the A_{260}:A_{280} ratio is <2.0, protein contamination is probable and re-extraction with phenol is recommended. If the A_{260} is high, phenol contamination is probable and another round of ethanol precipitation and resuspension is recommended. In addition, the use of A_{260}:A_{230} ratio as an indicator of nucleic acid purity, with ratios commonly in the range of 2.0-2.2. Note that absorbance is pH dependent, so for accurate readings, keep the pH constant and near 7.5.

2.14.4 cDNA synthesis

Synthesize first-strand cDNA using M-MLV RT.

A 20-μL reaction volume was used for 1 ng–5 μg of total RNA.

The following components were added to nuclease-free microcentrifuge tube:

1. Oligo $(dT)_{12-18}$ (500 μg/mL), or 50–250 ng random primers, or 2 pmole gene-specific primer, 1 μL
2. Total RNA, 1 ng to 5 μg total RNA.
3. 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), 1 μL
4. The mixture was heated to 65^0 C for 5 minutes and quick chill on ice. The contents of the tube were collected by brief centrifugation.
5. Then we added:
   i. 5X First-Strand Buffer, 4 μL
   ii. 0.1 M DTT, 2 μL
   iii. RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/μL), 1 μL
6. The contents of the tube were mixed gently and incubated at 37°C for 2 minutes.
7. 1 μL (200 units) of M-MLV RT was added, and mixed by pipetting gently up and down. If using random primers, the tube was incubated at 25°C for 10 minutes.
8. Incubation for 50 minutes at 37°C.
9. The reaction was inactivated by heating at 70°C for 15 minutes.
10. The cDNA was be used as a template for amplification in PCR. However, amplification of some PCR targets (>1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, we added 1 μL (2 units) of E. coli RNase H and incubate at 37°C for 20 minutes.

2.14.5 Real-Time PCR

1. We performed duplicate PCRs per gene, per cDNA sample.

2. We prepared the following master mix, accounting for 2 additional reactions per gene.

Ingredient	Per reaction (µL)	For x reactions (µL)
RT² SYBR Green qPCR Master Mix	10	x X 10
Forward/Reverse primer mix (50 µM each)	1	x X 1
Molecular biology grade water	4	x X 4

3. The cDNA was diluted at 1:50 or 1:100 by first placing molecular biology grade water into a disposable sterile basin. 99 µl of the water was added into the PCR strip tube using a multichannel pipette.
4. 1 ml of cDNA was added to the labelled strip tubes using a multichannel pipette (e.g., Rainin L8-10).
5. The undiluted cDNA was recapped with new strip caps to prevent cross-contamination.
6. A fine tip marker was used to mark the plate to the location of the different master mixes.
7. The repeating pipette (Rainin, E12-20) was used to add 20 ml of master mix to each sample. One row at a time was used.
8. 5 µl of dilute cDNA was added to each of the wells using a 20 µL pipette.
9. The optical adhesive cover was used to seal the plate and centrifuged with a brief spin (up to 1,500 r.p.m.) on a centrifuge equipped with a 96-well plate adapter.
10. PCR was performed using the real-time instrument per the manufacturers' protocol. For Roche LightCycler the following conditions were used: 50^0 C for 2 min, 10 min at 95^0 C, and 40 cycles of denaturation at 950 C for 15 sec, and annealing /extension at 60^0 C for 30 sec. In each experiment no-template controls (NTC) and RT-minus controls were run in parallel to the experimental samples.

2.14.6 Primers designing

Primers were designed with Beacon Designer software (Premier Biosoft) in order to avoid template secondary structure and significant cross homologies regions with other gene by BLAST search. Primers used for real-time PCR are shown in the table 6

Table 6. Real time PCR Primers.

Gene	Forward primer	Reverse primer
BDNF	5'-CTGAGCGTGTGTGACAGTATTA-3'	5'-CTTTGGATACCGGGACTTTCTC-3'
NGF	5'-CAGTGAGGTGCATAGCGTAAT-3'	5'-CTCCTTCTGGGACATTGCTATC-3'
TrkA	5'-CAGTGATACCTGTGTCCATCAC-3'	5'-TTGTAGCACTCAGCGAGAAAG-3'
TrkB	5'- AAGGACTTTCATGGGAAGCTG-3'	5'- TCGCCCTCCACAACAGACAC-3'
p75	5'-GAGATGAGAGAGATCCGGAGAA-3'	5'- TGACCGAAGTCAAGGCATAAG-3'

VEGF	5'-TTGAGACCCTGGTGGACATCT-3'	5'-CACACAGGACGGCTTGAAGA-3'
iNOS	5'-GGAATCTTGGAGCGAGTTGT-3'	5'-CCTCTTGTCTTTGACCCAGTAG-3'
TNF-a	5'-TGTAGCCCACGTCGTAGCAA-3'	5'- AGGTACAACCCATCGGCTGG-3'
Arginase 1	5' AGCCAATGAAGAGCTGGCTGGT 3'	5'AACTGCCAGACTGGGTCT 3'

2.14.7 Data analysis using the ΔΔCT Method

The method of presenting quantitative real-time PCR data is the comparative CT method (also known as the $2^{-\Delta\Delta CT}$ method). The comparative CT method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene(320) .

Equation 1 shows the final form of the $2^{-\Delta\Delta CT}$ equation, the derivation of which has been reported previously in Applied Biosystems User Bulletin No. 2 (P/N 4303859) and according to Livak(320).

Equation 1: Fold change= $2^{-\Delta\Delta CT}$

This form of the equation may be used to compare the gene expression in two different samples (sample X and sample Y); each sample is related to an internal control gene (eg actin or GAPDH). Sample X may be the treated sample and sample Y, the untreated control; sample X may be the diseased state and sample Y, the normal state. Expanding Eq. 1 to its full form:

Equation 2: $2^{-\Delta\Delta CT}$= [(c_T of interest- c_T of internal control) sample X- (cT of interest- cT of internal control) sample Y)].

Quantitative gene expression using real-time PCR is always rely on PCR replicates (typically two to four PCR replicates per cDNA sample). Although there are several different approaches that could be taken, it is recommended to begin the analysis by taking the mean of these PCR replicates and then take the mean of the individual samples. It should be emphasized that statistical tests should not be run on the raw CT data and standard deviation should always be calculated after the $2^{-\Delta\Delta CT}$, $2^{-\Delta CT}$ or 2^{-CT} transformation has been performed(320).

2.15 Statistical analysis

P-values were calculated using Student's two tailed t-test when two conditions were compared and by one-way ANOVA followed by Tukey's post hoc tests. When normal distribution or equal variance assumptions were not valid, we used the non-parametric Mann Whitney t test or the unpaired t test with Welch's correction respectively. Error bars depict SEM. A P value of less than 0.05 was considered significant. Outliers were detected through Grubb's test. Sample size, number of replicates, and statistical tests are reported in figure legends and supplementary tables. Minimum sample sizes were determined a priori using power analyses or as dictated by the methodology (e.g. Flow-cytometry analyses).

2.16 Reagent setup

BrdU injection solution. Saline solution is warmed (0.9%w/v NaCl in sterile H_2O) to 40–50^0 C and BrdU is slowly dissolved in saline solution by gently vortexing. BrdU injection

solution is allowed to cool to room temperature (22–25^0 C) and the BrdU injection solution is used immediately.

PBS, 0.1 M, pH 7.4. In a 1–2 L beaker, we add 2.7 g of sodium phosphate monobasic NaH_2PO_4, 11.5 g of sodium phosphate dibasic Na_2HPO_4 and 9 g NaCl. Then, we add distilled water up to 1000 ml and stir. The pH should be around 7.4.

Blocking solution, 0.1 M PBS, 0.3% Triton X-100, 4% serum. In a 1–2 L beaker we add 40ml of serum, 3 ml of Triton X-100, 0.1MPBS up to 1,000ml and stir. We store the solution in 50-ml aliquots at –20^0 C.

Sodium citrate buffer, 10mM, 0.05 Triton X-100, pH 6. In a 1–2 L beaker we add 2.94 g of tri-sodium citrate (dihydrate) and distilled water up to 1,000 ml and stir. The pH is adjusted to 6.0 with 1 M HCl. Then, we add 0.5 ml of Triton X-100. Store at 4^0 C for up to 6 months.

4% Paraformaldehyde in 0.1 M PBS. In a 1–2 L beaker we add about 800 ml of 0.1M PBS and heat to 60–65^0 C while stirring. At 60–65^0 C, we add 40 g of paraformaldehyde powder slowly while stirring (note: adding a few drops of 1M NaOH helps to keep the solution clear). We continue to stir until the paraformaldehyde powder is dissolved, making sure that the temperature stays between 60 and 65^0 C. Finally, we cool the solution until it reaches room temperature. The solution is filtered and kept at 4^0 C. CAUTION! PFA is prepared in the fumehood.

Potassium Phosphate buffer 50 mM. In a 1 L beaker, we add solution B (K_2HPO_4, 8.7 g of dibasic potassium phosphate in 1 L of dH2 O) to solution A ($KH_2 PO_4$, 6.8 g of monobasic potassium phosphate in 1L of dH_2O) until a pH of 6.0 is achieved. Remaining solutions can be stored in fridge (2-8°C) until future use.

Hexadecyltrimethylammonium bromide (HTAB) buffer. In 1 L of Potassium Phosphate buffer (50 mM, pH=6.0) we add 5 g HTAB. Then, we gently heat to dissolve and store at 2-8°C until use.

3. Results

3.1 Acute DSS colitis enhances hippocampal neurogenesis

It is well-established that enhanced inflammatory responses in the periphery have a negative impact on hippocampal neurogenesis(321). To evaluate whether experimental acute colitis affects hippocampal neurogenesis, we utilized an established mouse model consisting of administration of DSS via the drinking water for 7 consecutive days(83). Oral administration of DSS induced strong colitis, characterized by significant weight loss (**Figure 1 a**), rectal bleeding (**Figure 1b**), elevated levels of tissue myeloperoxidase (MPO) (**Figure 1 c**), enhanced infiltration by mononuclear cells and loss of crypt structure in the colon (**Figure 1 d**).

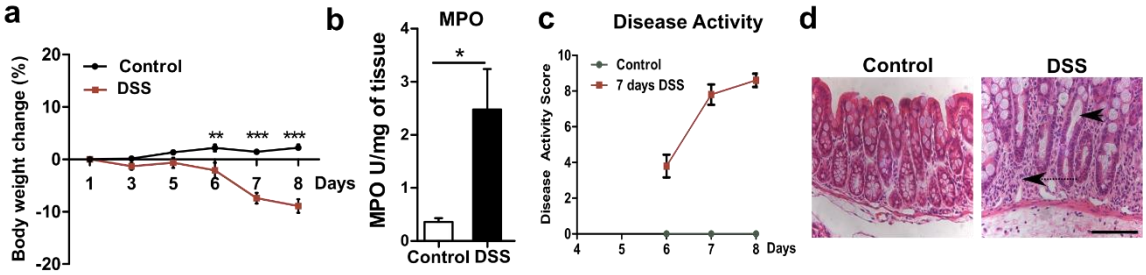

Figure 1. Evaluation of acute DSS colitis. *(a) Body weight changes (%) over the course of the disease. (b) Colonic myeloperoxidase (MPO) levels in the inflamed colonic tissue. (c) Disease activity score was calculated at the 6th day of acute DSS colitis were clinical signs were evident. (d) Representative H&E staining in the colon of control and mice with acute DSS colitis. Black arrowhead, crypt abscess; black dotted arrow, myeloid cell infiltrates. Scale bar: 10 mm. Each value represents the mean±SEM. Unpaired Student-test. n=8 mice/group from 2 independent experiments.*

To evaluate neurogenesis in the hippocampus, we used specific immunohistochemical markers for each stage of the hippocampal neurogenesis process(105). Hippocampal NSCs include a quiescent population with a radial glia-like morphology expressing the glial-like fibrillary acidic protein GFAP (Type 1), an activated intermediate progenitor cell type (Type 2a/2b) expressing Sox2 (SRY-related HMG-box gene 2) and a committed neuronal progenitor cell type, called neuroblast (Type 3), expressing doublecortin (DCX)(41). To our surprise, we observed that acute DSS colitis induced a marked increase in the numbers of GFAP$^+$/Ki67$^+$ cells with a radial-glial like morphology (P=0.0124; **Figure 2 a and c**), as well as, of the Sox2$^+$ cells (P=0.0033; **Figure 2 a and c**) and DCX$^+$ cells (P=0.0074; **Figure 2 B and c**) in the DG, compared to control mice, along with increased numbers of proliferating (Ki67$^+$) progenitor cells (P=0.0041; **Figure 2 a and c**). These findings suggest that acute DSS colitis induces activation of the quiescent pool of NSCs (GFAP+/Ki67+), along with increases in fast proliferating progenitor cells (Sox2+) and neuroblasts (DCX+).

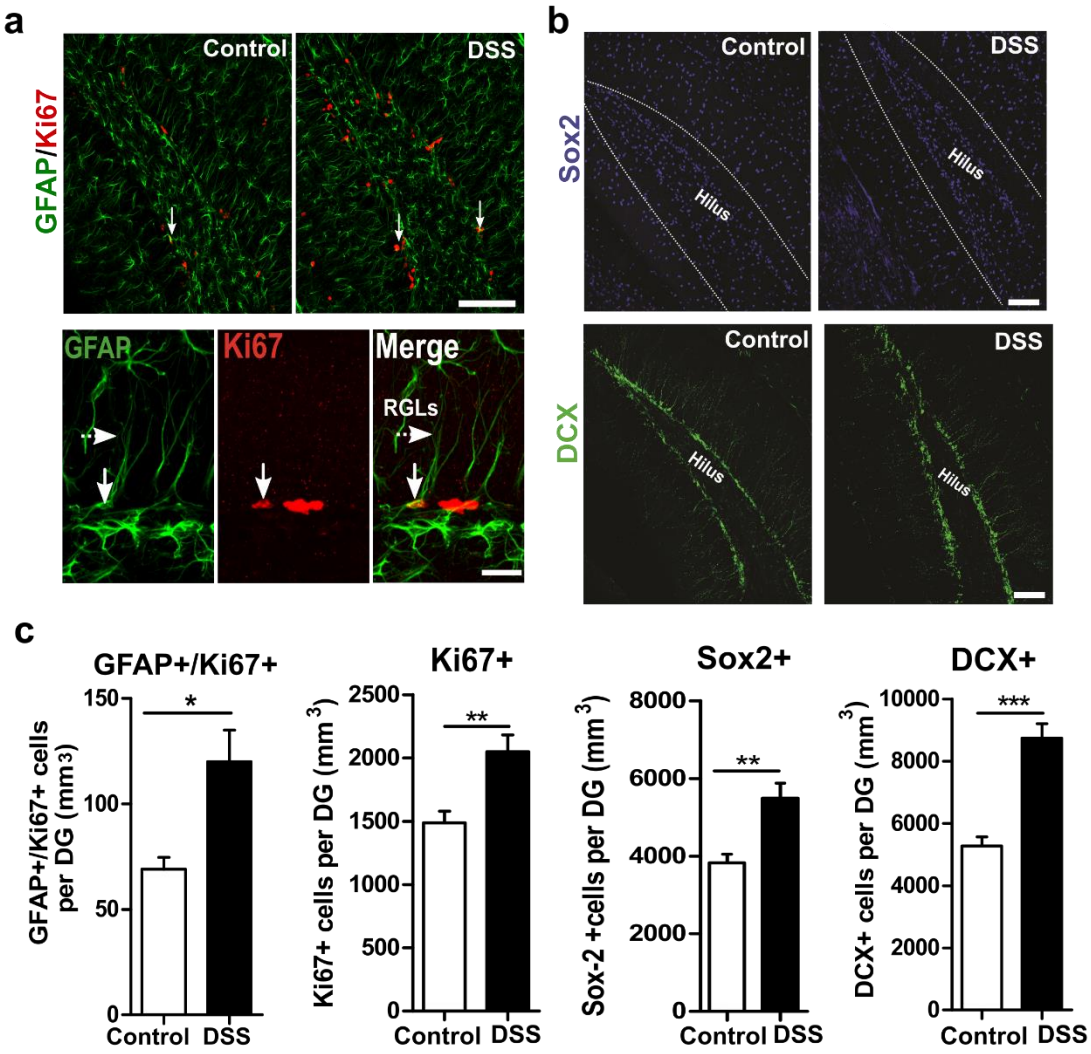

Figure 2. Acute DSS colitis induces hippocampal neurogenesis. *(a) Representative confocal maximal projection images of GFAP$^+$/Ki67$^+$ cells in the DG. Scale bar: 100 µm. Higher magnification images of GFAP$^+$/Ki67$^+$ cells with radial glial like morphology. Arrows depict GFAP$^+$/Ki67$^+$ cells in the SGZ. Scale bar: 20 µm. (b) Representative confocal maximal projection images of Sox2$^+$ and DCX$^+$ cells in the DG. Scale bar: 100 µm. (c) Stereological quantification of GFAP$^+$/Ki67$^+$, Sox2$^+$, DCX$^+$ cells in the DG. Each value represents the mean±SEM. 2 independent experiments (n=8). Student unpaired t-test. (***P<.001, **P<.01, * P<.05). RGLs: Radial Glial like cells.*

3.2 Acute DSS colitis induces altered cell cycle kinetics in proliferating neural progenitor cells in the hippocampal DG

To further validate the aforementioned findings, we administered BrdU at the 7th day of DSS colitis, to mark neuronal cells entering the S-phase of the cell cycle (Experimental setup**; Figure 3 a)**. Interestingly, following 24 hours of BrdU incorporation, the numbers of total BrdU$^+$ cells did not significantly differ between the two experimental groups (*P*=0.5471; **Figure 3 b**). Still, we noticed that the numbers of BrdU$^+$/GFAP$^+$/Sox2$^+$ cells, corresponding to Type 1 cells, were increased (*P*=0.0143; **Figure 3 b-d**) in the DG of mice with DSS colitis, compared to control mice. Similarly, the numbers of BrdU$^+$/Sox2$^+$/DCX$^+$ cells (Type

2b) were also significantly elevated (P=0.0387; **Figure 3 b, e and f**). In contrast, the numbers of $BrdU^+/Sox2^+/GFAP^-/DCX^-$ cells (*P*=0.0053; **Figure 3 b-d**) and those of $BrdU^+/Sox2^-/DCX^+$ cells (*P*=0.0228; **Figure 3 b, e and f**) that identify Type 2a and Type 3 cell types respectively, were significantly decreased.

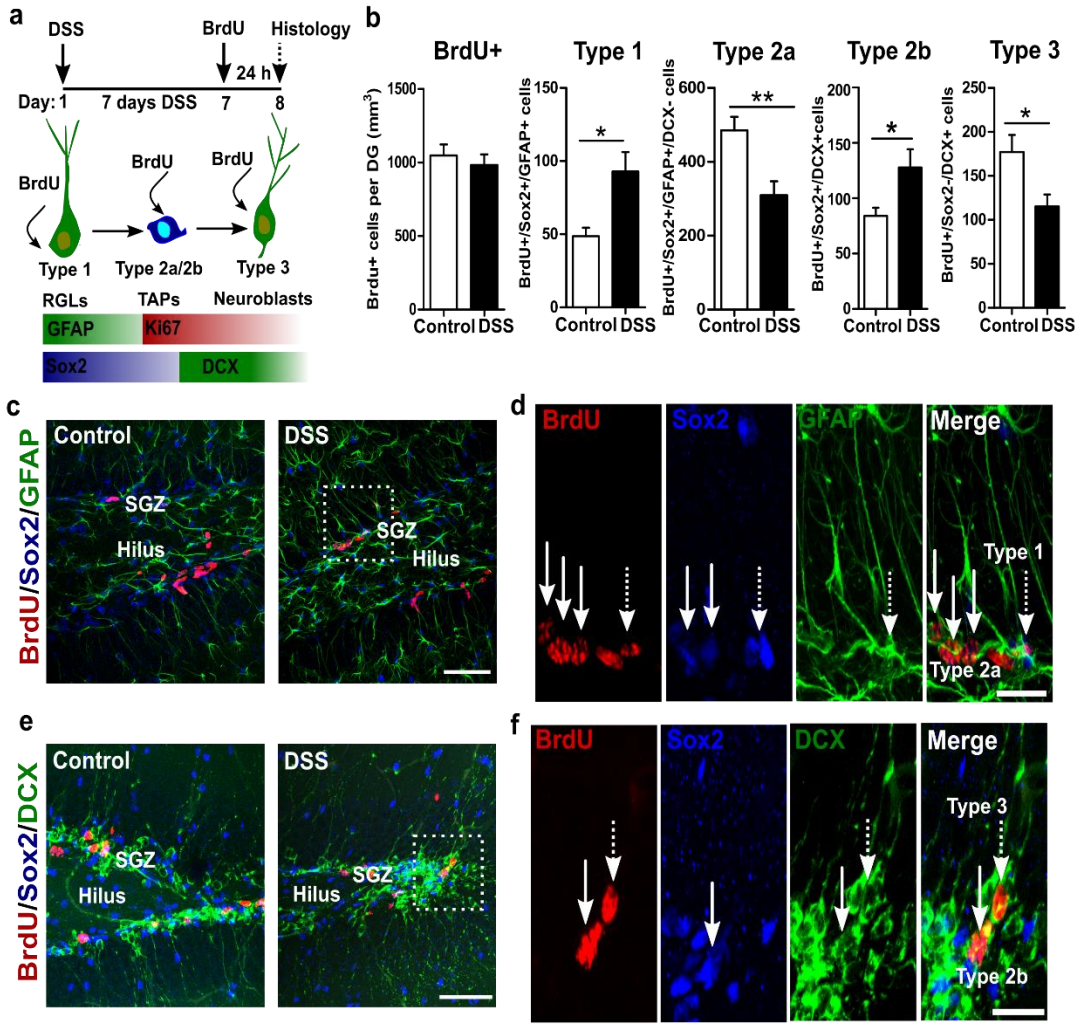

Figure 3. Acute DSS colitis affects the proliferation of hippocampal NSCs. *(a) Experimental setup with schematic representation of the hippocampal neurogenesis process, including specific cell types and their specific markers. (b) Stereological quantification of total BrdU+ cells, Type 1, Type 2a, Type 2b and Type 3 cells in the DG of control and mice with acute DSS colitis (n=8). (c) Representative triple-labelled confocal maximal projection images of BrdU with GFAP and Sox2. Scale bar: 50 µm. (d) Higher magnification micrographs (from box) of triple-labelled maximal projection images of BrdU with GFAP and Sox2-expressing cells. Thick white arrows depict Type 2a cells and dotted white arrows Type 1 cells. Scale bar: 20 µm. (e) Representative triple-labelled confocal maximal projection images of BrdU with Sox2 and DCX. Scale bar: 50 µm. (f) Higher magnification micrographs (from box) of triple-labelled maximal projection images of BrdU with Sox2 and DCX-expressing cells. Thick white arrows depict Type 2b cells and dotted white arrows Type 3 cells. Scale bar: 20 µm. Each value represents the mean±SEM obtained from two independent experiments (n=7-10). unpaired Student t-test with Welch's correction when*

*appropriate for unequal variances. (**P<0.01, * P<0.05). RGLs: Radial glia-like stem cells, TAPs: transient amplifying progenitor cells.*

The aforementioned increase in the number of $Ki67^+$ cells, points to altered cell cycle kinetics in the NSCs of the hippocampus during acute DSS colitis. To explore this hypothesis, we evaluated cells positive for both Ki67 and BrdU markers, employing a well-established protocol that identifies cells that exit and re-enter the cell cycle(322) (**Figure 4 a**). Notably, mice with acute DSS colitis presented with a significantly higher proportion of cells exiting the cell cycle (*P*=.0068; **Figure 4 b and c**) and significantly lower numbers of cells re-entering the cell cycle in the DG (*P*=.0074; **Figure 4 b and c**), suggesting that the duration of the S-phase of the cell cycle of proliferating progenitors in the DG of mice is significantly reduced. This effect seems to be cell type-specific, as only Type 2a and Type 3 cells exhibited reduced numbers of $BrdU^+$ cells in the DG during acute DSS colitis.

Overall, these data suggest that acute DSS colitis enhances neurogenesis in the hippocampus but with alterations in the cell cycle kinetics of proliferating progenitors.

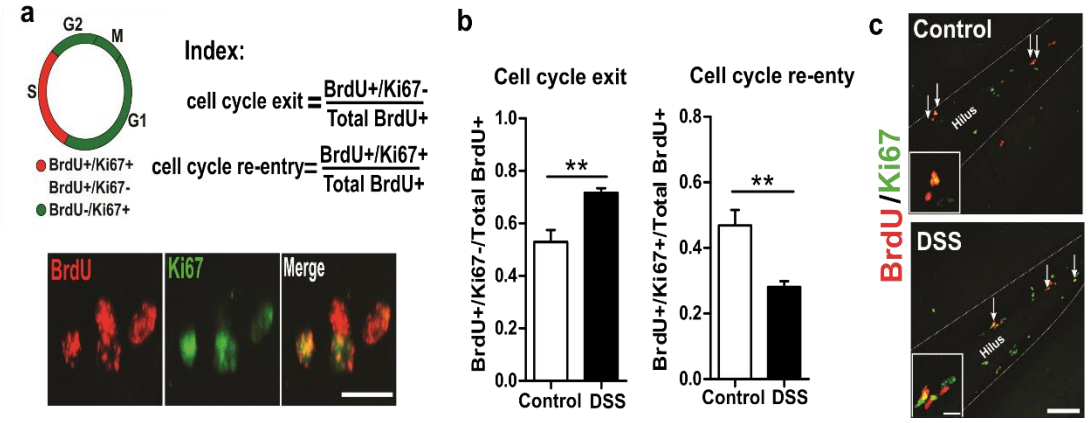

Figure 4. Acute DSS colitis induces deficits in cell cycle kinetics of proliferating hippocampal progenitors. *(a) Schematic representation of the cell cycle phases where BrdU and Ki67 co-labelling correspond to, and of the index used to indicate cell cycle exit and re-entry. Representative double- labelled confocal maximal projection images of BrdU+ with Ki67+ cells. Scale bar: 20 μm. (b) Quantification of cells exiting and re-entering the cell cycle in the SGZ of control and mice with acute DSS colitis (n=7-8). (c) Representative double- labelled confocal maximal projection images of BrdU+ with Ki67+ cells in the DG of control and mice with acute DSS colitis. Scale bar: 100 μm. Inset: higher magnification micrographs of double labelled BrdU+ and Ki67+ cells (arrows). Scale bar: 20 μm. Each value represents the mean±SEM obtained from two independent experiments (n=7-10). unpaired Student t-test with Welch's correction when appropriate for unequal variances. (**P<0.01, * P<0.05). RGLs: Radial glia-like stem cells, TAPs: transient amplifying progenitor cells.*

3.3 Acute DSS colitis induces apoptosis of hippocampal neuroblasts

Peripheral inflammation and their mediators (e.g cytokines) as well as activated microglia have been shown to induce apoptosis of neural stem cells in the DG(243,268).

To this extend, we evaluated the apoptosis of neural stem cells in the DG of control and mice with acute DSS colitis by staining with Caspase 3- a well described marker of apoptosis. Quantification of the number of Caspase 3^+ cells revealed significant increase in the SGZ of mice with acute DSS colitis (*P*=0.0003; **Figure 5 a and b**). Moreover, quantification of Caspase3^+/DCX$^+$ cells revealed an increased number of apoptotic neuroblasts in the SGZ of mice with acute DSS colitis (*P*=0.0270; **Figure 5 c and d**). These findings indicate that acute intestinal inflammation triggers the apoptosis of progenitors and neuroblasts in the DG.

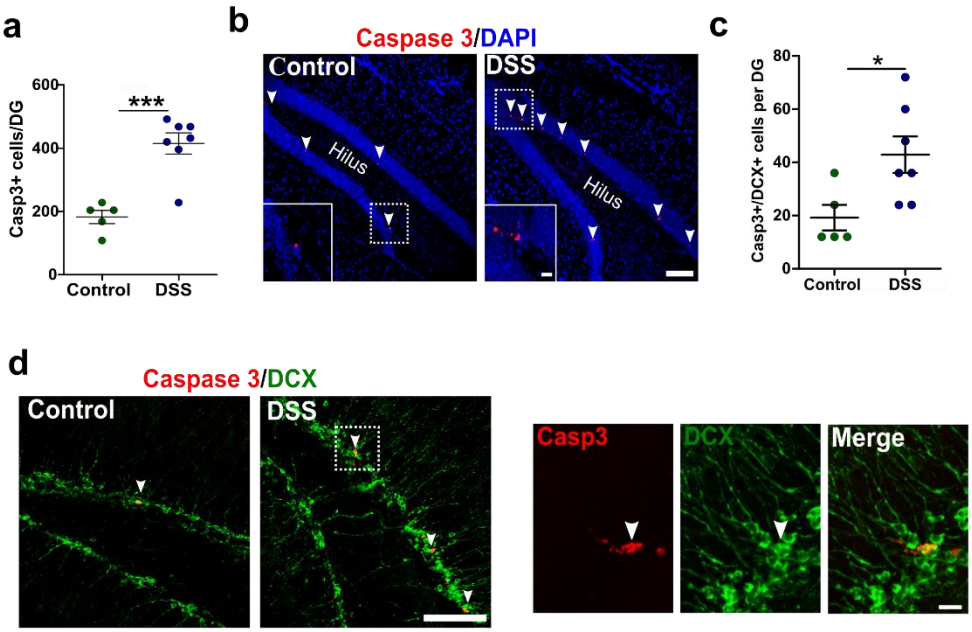

Figure 5. Acute DSS colitis induces apoptosis of neuroblasts in the DG. *(a) Stereological quantification of Caspase3+ cells in the SGZ of control and mice with acute DSS colitis. (b) Representative confocal maximal projection images of Caspase 3. Arrows depict the Caspase 3-expressing cells. Scale bar: 100 μm. Insets: Higher magnification images of Caspase3-expressing cells. Scale bar: 20 μm. (c) Stereological quantification of Caspase3+/DCX+ cells in the SGZ of Control and mice with acute DSS colitis. (d) Representative double labelled confocal maximal projection images of Caspase 3 and DCX. Higher magnification images of Caspase3-expressing cells. Arrows depict Caspas3+/DCX+ cells. Scale bar: 20 μm. Each value represents the mean±SEM obtained from two independent experiments (n=5-8). unpaired Student t-test. ***P<0.001, * P<0.05.*

3.4 Acute DSS colitis induces dissociation of the normal relation between proliferating NSCs and microvasculature

As we discussed previously, the region of hippocampus is highly vascularized and proliferating progenitor cells reside in close proximity to blood vessels which supply them with nutrients and signals maintaining homeostasis. Under pathological conditions such as neuroinflammation it has been shown that the normal relation between proliferating NSCs and blood vessels is disrupted(262).

Regarding the importance of vasculature in the neurogenesis process we proceeded to the measurement of the distance between the proliferating NSCs and the blood vessels in the SGZ. In mice with acute DSS colitis the average distance between dividing cells ($Ki67^+$) and the blood vessels stained with the vascular marker CD31 in the SGZ was found increased (Control: 3.138±0.2405 vs DSS: 5.706 ± 0.6108 μm; *P*=0.071, n=3-4; **Figure 6 a and b**). These data suggest that acute intestinal inflammation affected the vasculature compartment of the hippocampal neurogenic niche causing dissociation between endothelial cells of the blood vessels and active proliferating progenitors (**Figure 6 c**).

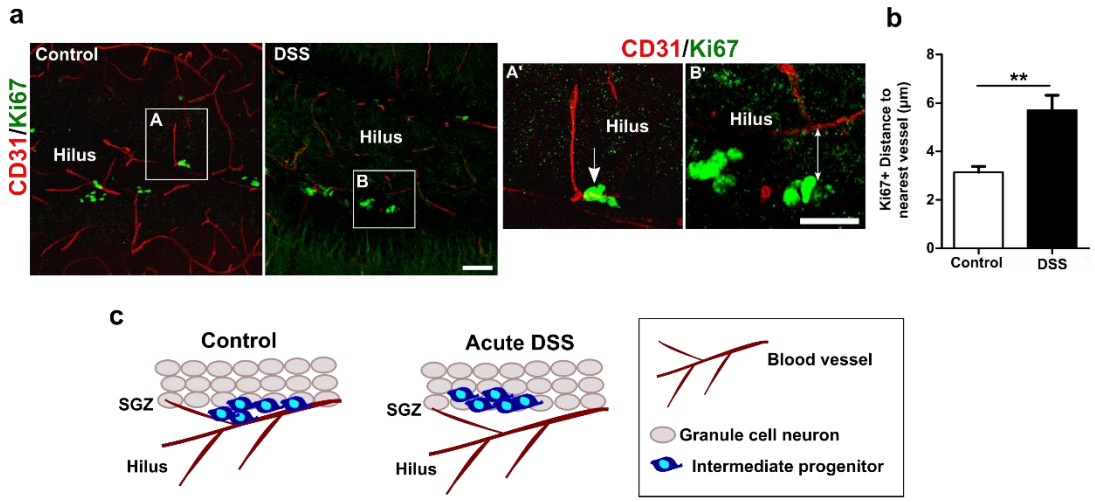

Figure 6. Acute DSS colitis induces dissociation between the vasculature and NSCs. *(a) Representative double labelled confocal maximal projection images of CD31 and Ki67. Higher magnification images (boxes) of CD31+ blood vessels and Ki67+ cells. Arrows depict Ki67+ cells. Scale bar: 20 μm. (b) Quantification of the distance between Ki67+ cells with the nearest CD31+blood vessel. (c) Schematic representation of the relation of proliferating NSCs/progenitors with blood vessels in SGZ of control and mice with acute DSS colitis. Each value represents the mean±SEM, (n=3-4). unpaired Student t-test. **P<.01.*

3.5 Hippocampal neurogenesis returned to baseline levels in Remission phase with a shift to astrocytic fate

Human IBD is characterized by repetitive acute phases of exacerbations followed by long periods of remission(33). In the DSS colitis model, remission phases are characterized by resolution of inflammation in the intestine with a gradually switch to a Th2-mediated response with decreased levels of TNF-α, IL-17 and elevated levels of IL-4,-6,-10 and IFNγ(80). Remission phase (1 cycle DSS) was characterized by excessive weight loss (**Figure 7 a**), elevated levels of MPO (**Figure 7 b**) and excessive infiltration by monocytes and loss of crypt structure in the colon (**Figure 7 c**).

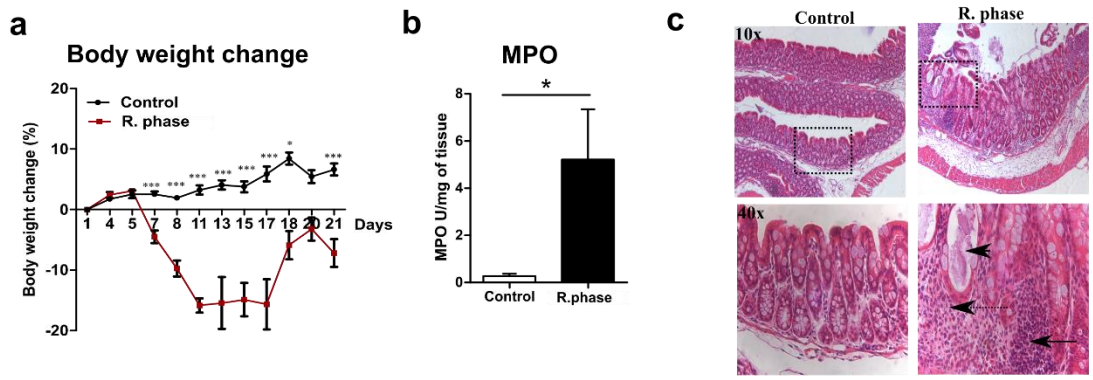

Figure 7. Evaluation of intestinal inflammation during the remission phase of DSS colitis. *(a) body weight changes (%) over the course of the disease. (b) Colonic myeloperoxidase (MPO) levels in the inflamed colonic tissue, and (c) Representative H&E staining in the colon of control and mice with acute DSS colitis. Black arrowhead, crypt abscess; black dotted arrow, neutrophilic infiltrates. Scale bar: 100 μm*

As previously, we evaluated neurogenesis in the hippocampus, using specific immunohistochemical markers for each stage of the hippocampal neurogenesis process. Interestingly, quantification of $Ki67^+$ (P=0.4459), $Sox2^+$ (P=0.4697) and DCX^+ (P=0.5687) cells revealed no differences in the DG of mice with remission phase compared to control (**Figure 8 a and b and Table 2**). Given the aforementioned increase in hippocampal neurogenesis during acute DSS colitis, these data indicate that during the remission phase, all cell types of the hippocampal neuronal lineage exerted apoptosis. More specifically, $Ki67^+$ cells were significantly decreased (~100 %) in the DG of mice with remission phase compared to mice with acute DSS colitis (P=0.0011; **Table 3**). Also, $Sox2^+$ and DCX^+ cells were decreased (~30 % and ~67% respectively) in the DG of mice with remission phase compared to mice with acute DSS colitis (P=0.0670 for Sox2 and P=0.0005 for DCX; **Table 3**).

Next, to assess the fate of hippocampal NSCs/progenitors during the remission phase we administered BrdU for 2 consecutive days after the induction of acute DSS colitis (8th and 9th day; **Figure 8 c**). Quantification of $BrdU^+$ cells revealed a slight but not significant increase in the SGZ of mice in remission phase (P>0.05; **Figure 8 d and f and Table 2**). Also, the number of $BrdU^+/NeuN^+$ cells that identify newborn neurons was similar in control and mice with remission phase (P>0.05; **Figure 8 d, e and f and Table 2**). However, when we quantified the number of $BrdU^+/GFAP^+$ cells that identify both activated RGLs and newborn astrocytes according to morphological criteria as previously described, we found a profound increase in the number of newborn astrocytes in the hilus near the SGZ (P=0.0009; **Figure 8 d, e and f and Table 2**). These results indicate that remission phase induces a shift

in the fate of hippocampal NSCs/progenitors to produce astrocytes without altering the number of newborn neurons.

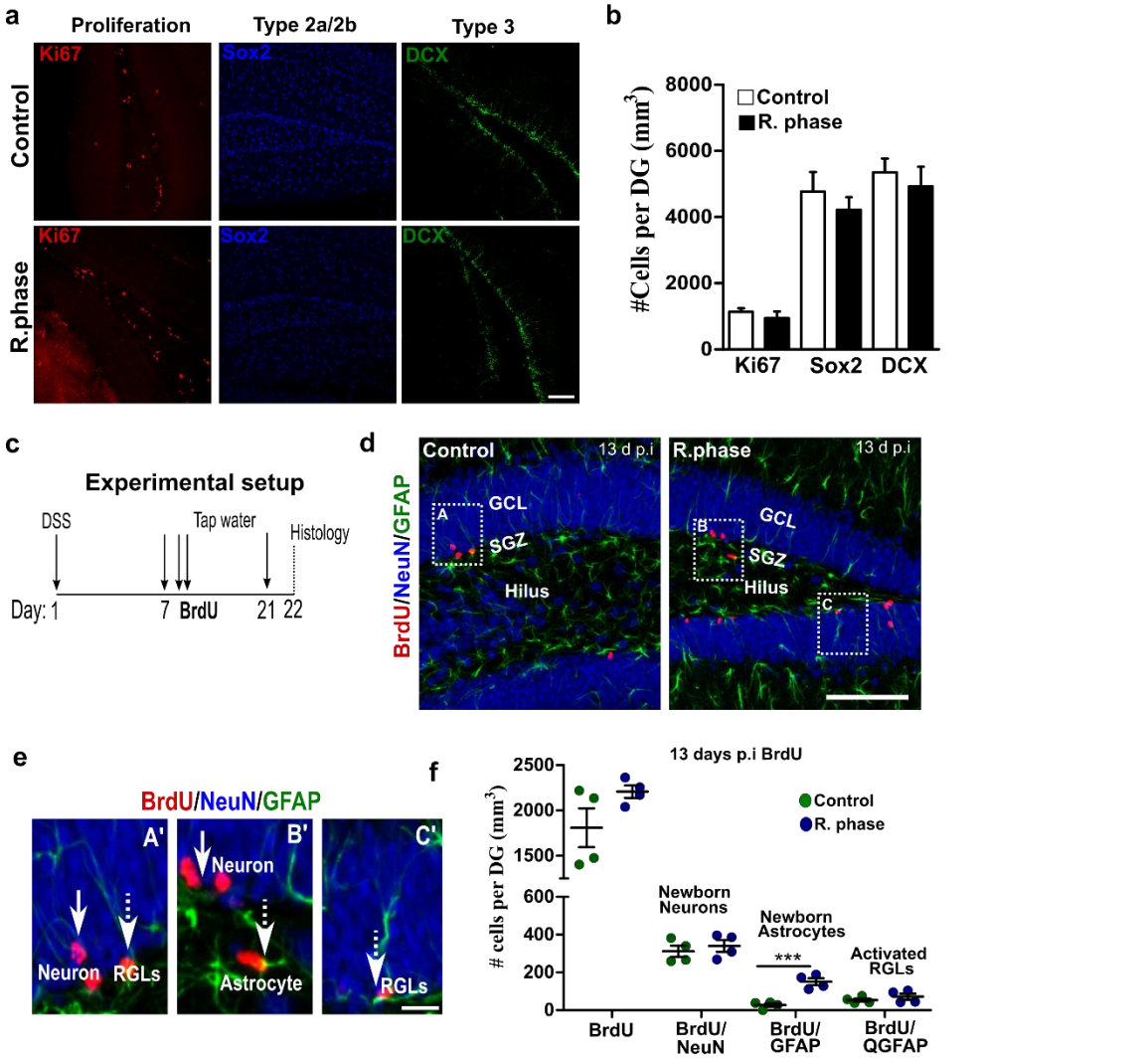

Figure 8. Hippocampal neurogenesis returns to baseline levels in Remission phase with a shift in astrogenesis. *(a) Representative confocal maximal projection images of Ki67, Sox2 and DCX staining in the DG of control and mice with remission phase. Scale bar: 100 μm. (b) Stereological quantification of Ki67$^+$, Sox2$^+$ and DCX$^+$ cells in the DG of control and mice remission phase. (c) Experimental setup. (d) Representative triple labelled confocal maximal projection images of BrdU with NeuN and GFAP in the DG of control and mice with remission phase. Scale bar: 100 μm. (e) Higher magnification images of BrdU$^+$/NeuN$^+$/GFAP$^+$ cells (boxes) where arrows depict newborn neurons (BrdU$^+$/NeuN$^+$) and dashed arrows newborn astrocytes (BrdU$^+$/GFAP$^+$) with the characteristic astrocytic morphology and activated RGLs (BrdU$^+$/GFAP$^+$) with the characteristic long process through the GCL. Scale bar: 10 μm. (f) Stereological quantification of BrdU$^+$, BrdU$^+$/NeuN$^+$ and BrdU$^+$/GFAP$^+$ cells in the DG of control and mice with remission phase. Each value represents the mean±SEM, (n=4-9). unpaired Student t-test. ***P<0.001.*

3.6 Chronic DSS colitis affects the migration of newborn neurons in the hippocampal DG

As human IBD is a chronic inflammatory condition with cycles of relapses and remissions(323), we next assessed the effects of chronic DSS colitis on the survival and differentiation of the newborn hippocampal DG neurons. To address this, we applied the well-established, multiple cycles of DSS administration protocol that induces a gradually worsening, rather than self-limiting, form of colitis that recapitulates the relapsing-remitting nature of human IBD(83). As previously described, upon three cycles of DSS administration(83), development of long-lasting colitis with characteristic clinical disease features, such as, weight loss (**Figure 9 a**), rectal bleeding and diarrhea (**Figure 9 b**) was observed. Moreover, we found endpoints relevant to the human disease, such as, elevated levels of MPO (P<0.0001) (**Figure 9 c**), profound infiltration by mononuclear cells in the affected tissue (**Figure 9 d**) and significantly decreased length of the colon (P<0.0001), as compared to control mice (**Figure 9 e**).

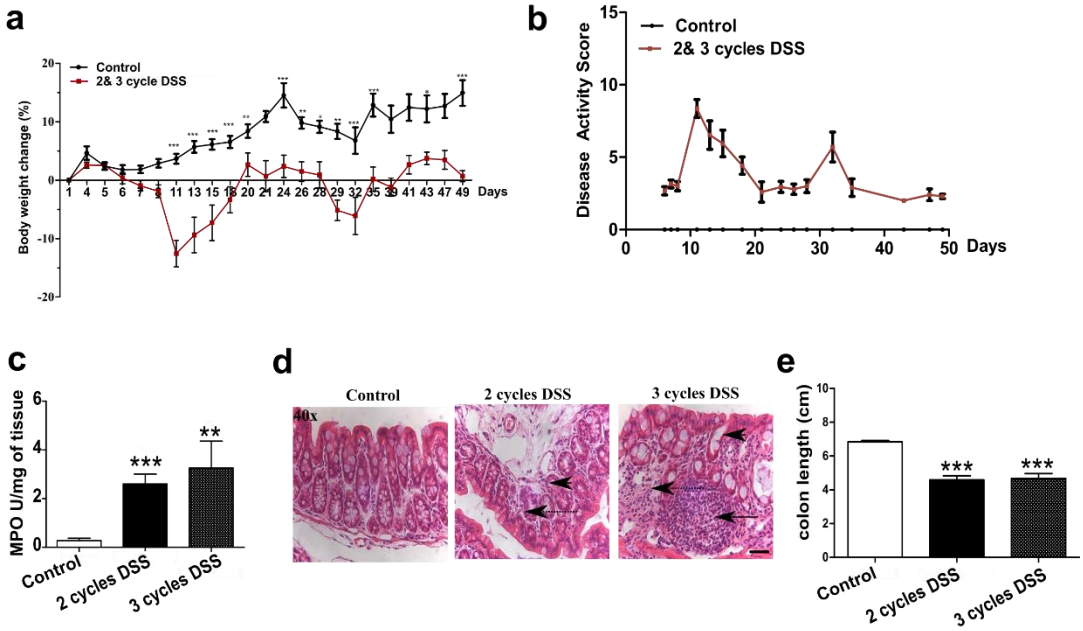

Figure 9. Establishment and evaluation of chronic DSS colitis. *(a) Cumulative change of body weight in the course of Chronic DSS colitis from 2 independent experiments. n= 8 mice/group. (b) Disease activity score was calculated through the course of chronic DSS colitis. (c) Colonic myeloperoxidase (MPO) levels are increased in chronic DSS colitis (Control: n= 8, 2 cycles DSS: n=9, 3 cycles DSS: n= 9). (d) Representative images of H&E staining in the colon of control and mice with chronic DSS colitis. Black arrowhead, crypt abscess; black dotted arrow, mononuclear infiltrates; black arrow, area of strong transmural inflammation with loss of crypt structure and depletion of goblet cells. (e) Colon length was decreased in mice with chronic DSS colitis (Control: n= 18, 2 cycles DSS: n=6, 3 cycles DSS: n= 9). Each value represents the mean±SEM. Two-way ANOVA with Bonferroni's post hoc test was performed for Body weight change. One way ANOVA with Bonferroni's post hoc test was performed for Colon length and MPO levels. (***P<0.001, **P<0.01, * P<0.05). Scale bar: 100 μm*

To trace the newborn neurons in the DG of mice with chronic DSS colitis (3 cycles of DSS), we administered BrdU for 3 consecutive days before the first cycle of DSS administration (**Figure 10 a**). This experimental strategy enabled us to evaluate the survival, differentiation and migration of DG newborn neurons throughout the course of chronic colitis(309). Interestingly, in line with our findings in acute colitis, the total numbers of $BrdU^+$ cells, and of the immature ($BrdU^+/DCX^+/NeuN^+$) and mature ($BrdU^+/DCX^-/NeuN^+$) neurons, were not significantly altered in mice with chronic colitis ($P>0.05$; **Figure 10 b and c**), suggesting that chronic intestinal inflammation did not affect the survival of these, newly-generated, hippocampal neurons. Moreover, chronic DSS colitis did not alter the numbers of proliferating progenitor cells ($P>0.05$; $Ki67^+$ cells; **Figure 10 d**) and neuroblasts in the hippocampal DG ($P>0.05$; DCX^+ cells; **Figure 10 e**). Similar findings were obtained in mice subjected to 2-cycles of DSS administration, further supporting the notion that repeated cycles of DSS do not affect the generation and survival of new hippocampal dentate granule neurons (**Figure 10 f and g**).

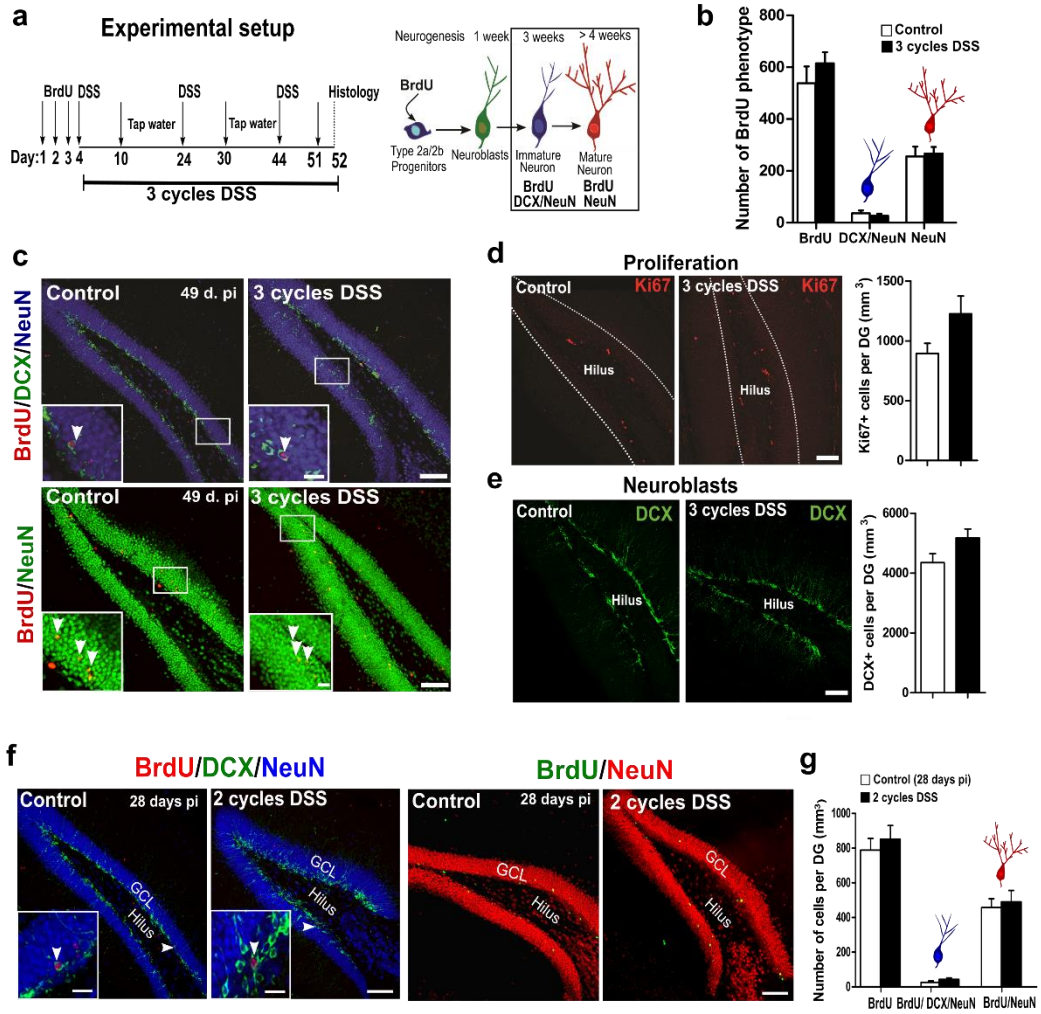

Figure 10. Chronic DSS colitis does not affect the number of newborn neurons in the DG. *(a) Experimental setup with schematic representation of the hippocampal neurogenesis process, including the specific markers of immature and mature newborn neurons. (b) Stereological*

quantification of total BrdU$^+$ cells, immature (BrdU$^+$/DCX$^+$/NeuN$^+$) and mature newborn (BrdU$^+$/NeuN$^+$) neurons in the DG of control and mice with chronic DSS colitis. (c) Representative double and triple- labelled confocal maximal projection images of BrdU$^+$ with DCX$^+$ and NeuN$^+$ cells in the DG of control and mice with chronic DSS colitis. Scale bar: 100 μm. Inset: higher magnification micrographs of double and triple- labelled BrdU$^+$ with DCX$^+$ and NeuN$^+$ cells (arrows). Scale bar: 20 μm.(d) Representative confocal maximal projection images of Ki67$^+$ cells in the DG of control and mice with chronic DSS colitis (3 cycles). Scale bar: 100 μm. Stereological quantification of Ki67$^+$ cells in the DG of control and mice with chronic DSS colitis (3 cycles) (n=5). (e) Representative confocal maximal projection images of DCX$^+$ cells in the DG of control and mice with chronic DSS colitis (3 cycles). Scale bar: 100 μm. Stereological quantification of DCX$^+$ cells in the DG of control and mice with chronic DSS colitis (3 cycles) (n=6). (f) Representative confocal maximal projection images of BrdU$^+$/DCX$^+$/NeuN$^+$ and BrdU$^+$/DCX$^-$/NeuN$^+$ cells in the DG of control and mice with chronic DSS colitis (2 cycles). Scale bar: 100 μm. Higher magnification micrographs (boxes) of maximal projection images of cells triple-labelled with BrdU, DCX and NeuN. Arrows depict BrdU/DCX/NeuN-expressing cells. Scale bar: 20 μm. (g) Stereological quantification of BrdU$^+$, BrdU$^+$/DCX$^+$/NeuN$^+$ and BrdU$^+$/DCX$^-$/NeuN$^+$ cells in the DG of control and mice with chronic DSS colitis (2 cycles) (n=7-9). Each value represents the mean±SEM from 2 independent experiments. Unpaired Student t-test. P>0.05. GCL: Granule cell layer.

Adult newborn neurons migrate a short distance into the GCL of the DG and functionally integrate in the hippocampal circuitry(105). Conditions, such as, traumatic brain injury (324)or LPS-mediated chronic neuroinflammation(229), significantly alter the migration pattern of newly generated neurons. Notably, analyses of the migration pattern of newborn neurons during chronic colitis revealed significant defects (**Figure 11 a**). More specifically, the average index of migration of BrdU+/NeuN+ cells through the GCL of the DG in mice with chronic DSS colitis was significantly lower compared to the control group (Control: 30.49±1.36 vs 3 cycles DSS: 20.72±1.06 μm; P<0.0001; **Figure 11 b and d**), while no differences were detected in the DG volume based on assessment of the width of GCL (P>0.05; **Figure 11 c**). Taken together, these findings suggest that although chronic DSS colitis does not affect the overall survival and production of newly generated DG neurons, it significantly impacts their migration patterns.

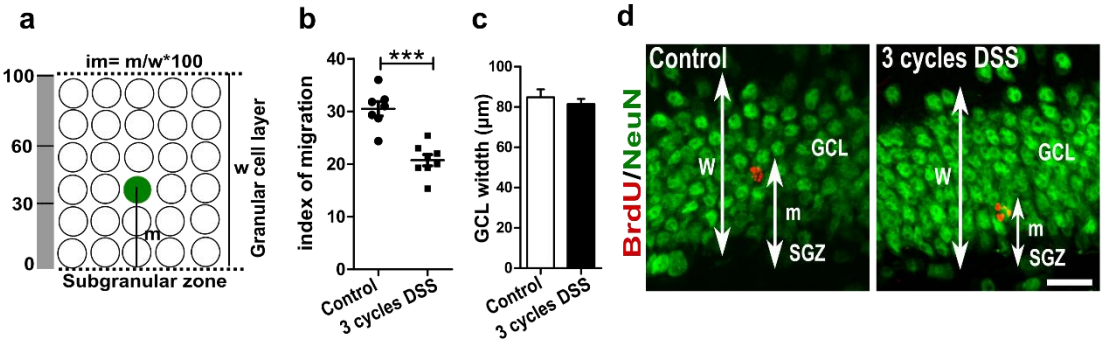

Figure 11. Chronic DSS colitis affects the migration of newborn neurons in the DG. *(a) Schematic representation of the calculation method of the migration of newborn neurons through the GCL. Index of migration calculated using the formula im= m/w*100, where m is the distance between the center of BrdU$^+$/NeuN$^+$ cells and the subgranular zone; w is the width of granule cell layer. (b) The index of migration was lower in mice with chronic DSS colitis. (n=20 cells/mouse, n=8). (c) GCL width in control and mice with chronic DSS colitis. (d) Representative confocal maximal projection images depicting the migration pattern of BrdU$^+$/NeuN$^+$ cells through the GCL in control and mice*

*with chronic DSS colitis. Scale bar: 20 μm. Each value represents the mean±SEM obtained from n=8 mice/group, 2 independent experiments. Unpaired Student t-test (***P<0.0001, **P<0.01, *P<0.05). SGZ: Subragranular zone, GCL: Granule cell layer.*

3.7 Chronic DSS colitis affects the integration of newborn neurons in behaviorally-relevant hippocampal networks

Migration deficits may alter the integration of newborn neurons, since their abnormal position inside the GCL affects their function(270). Indeed, previous studies using the immediate early gene Arc as a marker of neuronal activity, showed that recruitment of 4-6-week-old adult newborn neurons in the DG in response to a novel spatial environment, is significantly compromised under neuroinflammatory conditions(229,325). Hence, we next hypothesized that migration deficits observed in the context of chronic colitis, may affect the integration of newborn neurons in the hippocampus and, as such, induce functional impairment. To explore this possibility, we utilized a well-established behavioral paradigm (326) and assessed whether 7 week-old newborn neurons were functionally integrated into the DG circuitry of mice with chronic colitis. Briefly, control and mice administered DSS for 3 cycles were exposed to a novel spatial environment for 5 minutes in two exploration sessions. Between the two exploration sessions there was an interval of 30 minutes where mice were left undisturbed in the home cage. Interestingly, we found that mice with chronic colitis were hypoactive, as indicated by their moving speed (P<0.01; **Figure 12 a**) and the corresponding distance covered (P<0.01; **Figure 12 a**), during the 1st exploration session, compared to the control group (**Figure 12 a**). As expected, in the 2nd exploration session, control mice decreased their exploration activity, as indicated by the overall distance covered and velocity (P<0.01; **Figure 12 a**), suggesting that mice became accustomed to the novel spatial environment. In sharp contrast, mice with chronic colitis covered the same distance, time in zone and velocity in the 2nd exploration session, compared to those observed during the 1st exploration session (**Figure 12 a**). These findings are indicative of either spatial learning deficits or impaired mobility due to their disease.

In support of the former, we assessed the expression of Arc by immunohistochemistry and observed a significant decrease in Arc+ cells throughout the DG in mice with chronic DSS colitis, as opposed to the induction of Arc expression in the DG neurons of control mice (Control: 1493±137.4 vs 3 cycles DSS: 878±101.0 cells, n=5-6; P=0.0050; **Figure 12 b and c**). Moreover, the percentages of mature granule neurons expressing Arc were also decreased in the DG of mice with chronic DSS colitis (Control: 3.445±0.303 vs 3 cycles DSS: 2.220±0.2786 %, n=5-6; P=0.0153; **Figure 12 d**).

Next, in order to analyze the percentages of newborn neurons expressing Arc in response to spatial exploration, we performed triple staining for BrdU, NeuN and Arc (**Figure 12 e**). As expected based on our findings above, the numbers of newborn neurons were not different in the two experimental groups (P>0.05; **Figure 12 f**). However, the proportion of BrdU-labeled newborn neurons expressing Arc was significantly higher in the control group compared to the mice with chronic colitis (Control: 4.23 ±1.23018 % vs chronic DSS: 1.212±0.767 %; P=0.0444; n=5-6; **Figure 12 e and g**). Taken together, these data provide evidence that mice with chronic colitis demonstrate impaired ability to respond to a novel

spatial environment and exhibit dysregulated Arc expression in both mature and newborn dentate granule neurons.

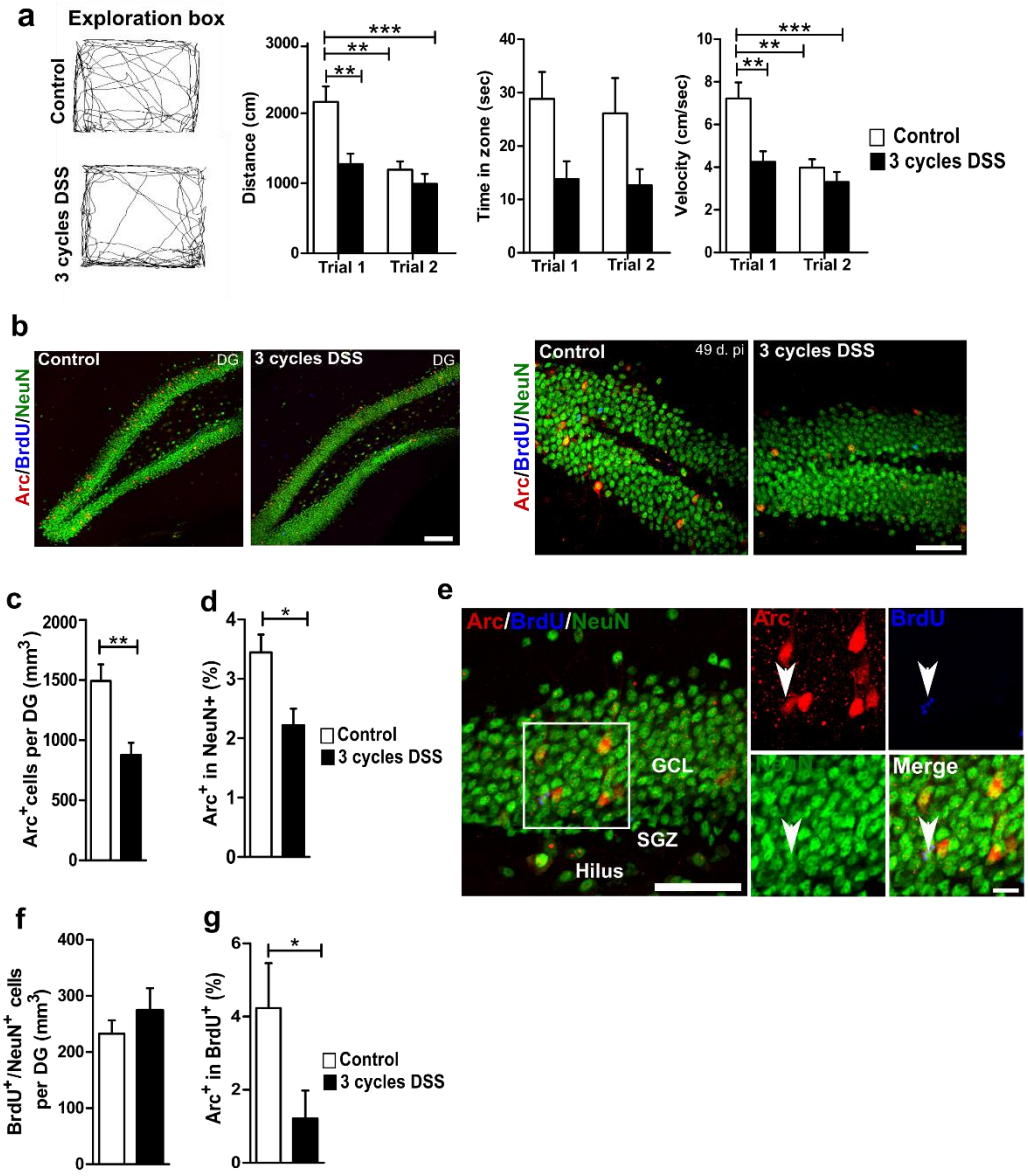

Figure 12. Chronic DSS colitis affects the integration of newborn and mature dentate granule neurons in the functional circuitry of the DG. *(a) Control and mice with chronic DSS colitis were placed to explore freely a novel spatial environment (box) for 5 minutes with a 30 minutes interval intervening between the first and second exploration session. Distance (cm) covered, time in zone (s) and velocity (cm/s) were calculated in both experimental groups (n=7). Statistical significant was obtained by One way ANOVA followed by Tukey's post hoc test (***P<0.001, **P<0.01). (b) Representative triple- labelled confocal maximal projection images of BrdU$^+$ with Arc$^+$ and NeuN$^+$ cells in the DG of control and mice with chronic DSS colitis. Scale bar: 50 μm. (c) Stereological quantification of total Arc$^+$ cells in the DG of control and mice with chronic DSS colitis (n=5-6). (d) Percentages of mature neurons (NeuN$^+$) expressing Arc in the whole DG of hippocampus of control and mice with chronic DSS colitis (n=5-6). (e) Representative triple- labelled confocal maximal projection images of BrdU$^+$ with Arc$^+$ and NeuN$^+$ cells in the DG. Scale bar: 50 μm. Higher magnification micrographs (from box) of triple-labelled maximal projection images of BrdU with Arc and NeuN-expressing cells (arrows). Scale bar: 20 μm (f) Stereological quantification of BrdU$^+$/NeuN$^+$ in the DG of control and mice with chronic DSS colitis (n=5-6). (g) Percentages of*

*newborn neurons ($BrdU^+/NeuN^+$) expressing Arc in the whole DG of hippocampus of control and mice with chronic DSS colitis (n=5-6). Each value represents the mean±SEM. Unpaired Student t-test with Welch's correction when appropriate for unequal variances (***P<0.001, **P<0.01,*P<0.05). n=5-7 mice/group. SGZ: Subragranular zone, GCL: Granule cell layer*

3.8 Dissociation of the normal relation between proliferating NSCs and microvasculature is still present in mice with chronic DSS colitis

Acute DSS colitis induced dissociation of the normal relation between actively proliferating progenitors and the microvasculature of the hippocampal neurogenic niche. This effect was still evident in the SGZ of mice administered with DSS for 2 cycles (Control: 4.521 ± 0.5000 μm, 2 cycles DSS: 7.549 ± 0.6325 μm, *P*=0. 0002, n=4; **Figure 13 a and b**) and 1 3 cycles (Control: 5.587± 0.3481 μm, 3 cycles DSS: 8.357 ± 0.4774 μm, *P*<0. 0001, n=4; **Figure 13 c and d).** These data suggest that acute colitis induced detrimental effects on the microvasculature of the hippocampal niche which were still evident in chronic DSS colitis. This dissociation of the relation of the microvasculature and proliferating progenitors could potentially lead to differentiation, maturation and migration deficits of NSCs which might explain the observed migration and integration deficits of newborn neurons in the DG of mice with chronic DSS colitis.

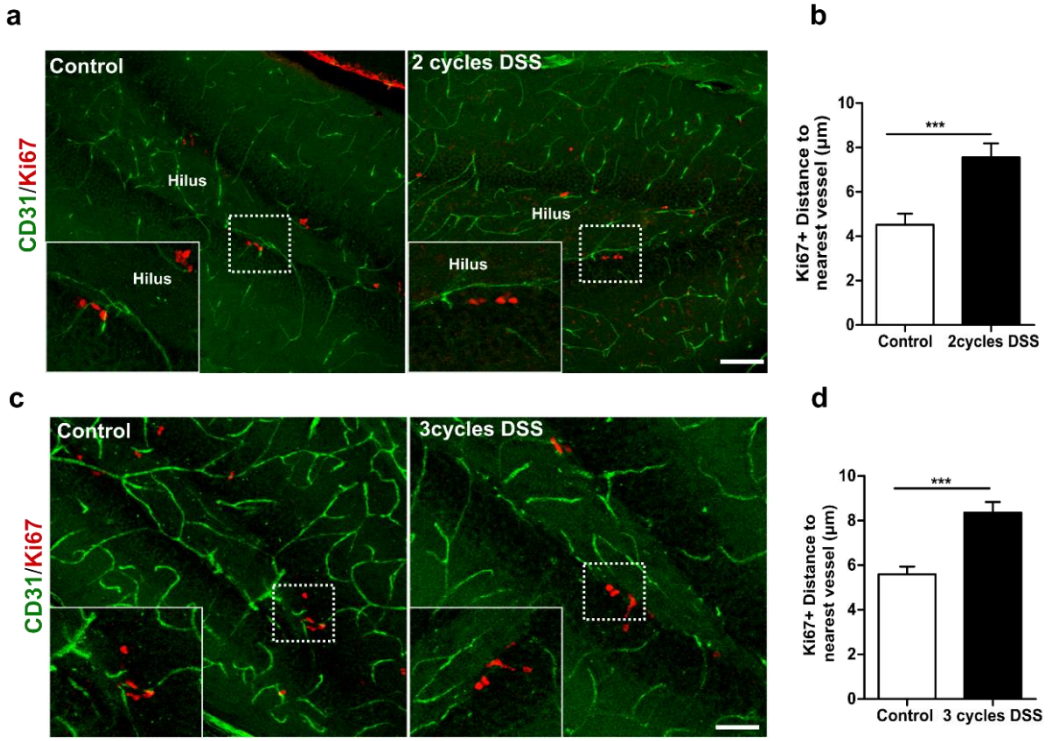

Figure 13. Dissociation between the vasculature and NSCs persists in chronic DSS colitis. *(a) Representative double labelled confocal maximal projection images of CD31 and Ki67. Higher magnification images (boxes) of CD31+ blood vessels and Ki67+ cells. Scale bar: 100 μm. (b) Quantification of the distance between Ki67+ cells with the nearest CD31+blood vessel in control and mice administered DSS for 2 cycles. unpaired Student t-test. ***P<.001. n=4 (c) Representative double labelled confocal maximal projection images of CD31 and Ki67. (d) Quantification of the distance between Ki67+ cells with the nearest CD31+blood vessel in control and mice with chronic DSS colitis (3 cycles DSS). unpaired Student t-test. ***P<.001. n=4. Each value represents the mean±SEM.*

3.9 Chronic DSS colitis does not induce apoptosis in the DG

Persistent inflammatory stimuli from the periphery can trigger apoptosis in the hippocampal neurogenesis niche, apart from the activation of the resident microglia(188). Therefore, we quantified the apoptotic cells (Caspase 3^+) in the DG of mice with chronic DSS colitis. The quantification analysis revealed no differences in the number of apoptotic cells in the DG of mice with chronic DSS colitis (P>0.05; **Figure 14 a and b and Table 3**). These findings suggest that despite the persistent inflammatory stimuli coming from the periphery, there were no signs of increased apoptosis in the hippocampal neurogenic niche.

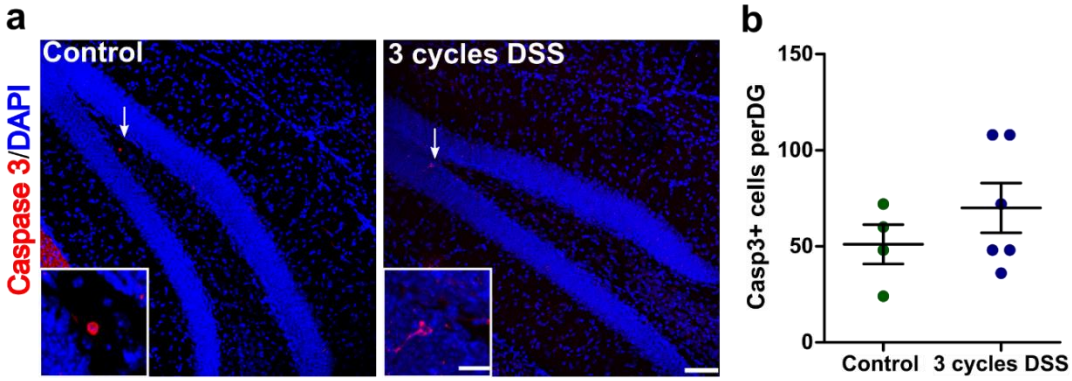

Figure 14. Chronic DSS colitis does not induce apoptosis in the DG. *(a) Representative confocal maximal projection images of Caspase 3. Higher magnification images (boxes) of Caspase 3 + cells. Arrows depict apoptotic cells. Scale bar: 100 μm. (b) Stereological quantification of Caspase 3+ cells in the SGZ of the DG of control and mice with chronic DSS colitis. Each value represents the mean±SEM. unpaired Student t-test. ***P<.001. n=4-7.*

3.10 Innate immune cells in the hippocampus exhibit distinct pro -and anti-inflammatory phenotypes during acute and chronic DSS colitis

Recent studies have highlighted a key role for innate immune cells in governing the communication between neural and immune pathways(327). Hence, we next explored the effects of the distinct phases of acute and chronic colitis on innate immune responses in the hippocampus.

To better understand the time-dependent changes in the activation of the innate immune response during colitis, we chose an earlier time point (day 4) following DSS administration, that does not coincide with the peak of the disease activity (**Figure 15 a, experimental setup**), as evidenced by the signs of inflammation including MPO release (P=0.0196) and mononuclear cell infiltration.

Interestingly, detailed immunophenotypic analyses with multi-color flow cytometry revealed increased percentages of infiltrating peripheral macrophages ($CD45^{high}CD11b^+$) in the hippocampus, as compared to control mice (Control: 0.6350 ± 0.08421 vs 4 days DSS: 1.608 ± 0.2610 %; *P=0.0121, n=4; **Figure 15 b**). In addition, we detected increased percentages of $CD45^+CD11b^+Ly6C^+$ cells, considered inflammatory myeloid cells in the hippocampus of mice with DSS colitis (Control: 1.888 ± 0.2061 vs 4 days DSS: 14.38 ± 0.9132 %; ***P<0.0001, n=4; **Figure 15 d and e**). Hippocampal infiltration by inflammatory myeloid cells ($CD45^+CD11b^+Ly6C^+$) and macrophages ($CD45^{high}CD11b^+$)

continued to increase, as shown by analyses on day 7 following DSS administration (Control: 1.888 ± 0.2061 vs 7 days DSS: 33.08± 1.817 %;***P<0.0001 for $CD45^{+}CD11b^{+}Ly6C^{+}$; Control: 0.6350 ± 0.08421 vs 7 days DSS: 3.720 ± 0.1818 % for $CD45^{high}$ $CD11b^{+}$; ***P<0.0001; n=4; **Figure 15 d and e**). In contrast, the percentages of infiltrating macrophages ($CD45^{high}CD11b^{+}$), and particularly those of inflammatory monocytes ($CD45^{+}CD11b^{+}Ly6C^{+}$), were decreased in the hippocampi of mice with chronic DSS colitis (2 cycles), compared to those with acute DSS colitis ($^{###}P$=0.0007 and $^{\&\&\&}P$<0.0001 for $CD45^{+}CD11b^{+}Ly6C^{+}$; **Figure 15 d, and e**). Altogether, these data indicate that during acute and chronic DSS colitis, hippocampus is infiltrated by circulating innate immune cells.

We next assessed the hippocampal microglial responses of mice on day 4 after DSS administration, that revealed increase in CD45lowCD11b+ cells (Control: 1.470 ± 0.1091 vs 4 days DSS: 2.975 ± 0.4230 %; *P=0.0137; n=4; **Figure 15 c**), that was even more prominent on day 7 (Control: 1.470 ± 0.1091 vs 7 days DSS: 9.553 ± 0.4352 %; ***P<0.001; n=4; **Figure 15 c**) and chronic DSS colitis (Control: 1.470 ± 0.1091 vs 2 cycles DSS: 7.143 ± 0.4252 %; ***P<0.001; n=4; **Figure 15 c**) compared to control mice.

Further characterization of the microglia immunophenotype demonstrated increased percentages of $CD45^{low}CD11b^{+}NOS2^{+}$ M1-like cells on day 4 (***P<0.0001; **Figure 15 f**), followed by significant decrease on day 7 ($^{(##}P$=0.0035; Figure 5 f). In contrast, the percentages of CD45lowCD11b+Arginase-1+ microglia with an M2-like phenotype, was markedly increased on day 7 (***P<0.0001 and $^{###}P$=0.0004; **Figure 15 g**). The switch in the immunophenotype of microglia was maintained during chronic colitis, as shown by the decreased numbers of M1-like cells ($^{##}P$=0.0013; **Figure 15 f**), and the elevated M2-like cells compared to early acute colitis ($^{##}P$=0.0091; **Figure 15 g**). Collectively, these data show that microglia responses in the hippocampus are distinct over the progress of colitis from the acute to its chronic phase.

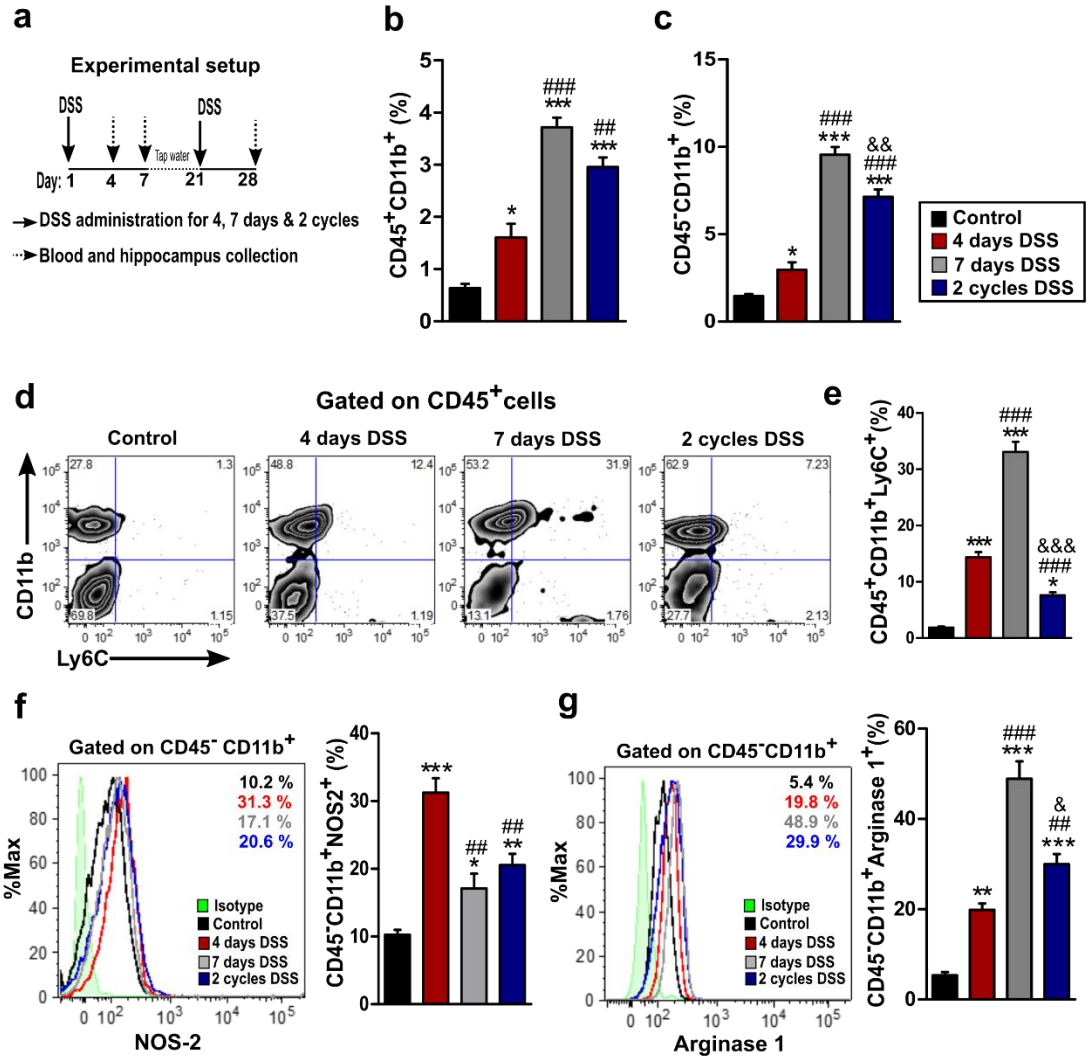

Figure 15. Innate immune cells in the hippocampus exhibit distinct phenotypes during acute and chronic DSS colitis. *(a) Experimental setup. DSS was administered for 4, 7 days and for 2 cycles. Blood and hippocampus were collected. (b) Percentages of $CD45^{+}CD11b^{+}$ (infiltrating macrophages) and (c) $CD45^{low/-}CD11b^{+}$ microglia in the hippocampus. (d) Representative FACS plots showing CD11b and Ly6c expression on $CD45^{+}$ cells. (e) Cumulative data showing the percentages of $CD45^{high}CD11b^{+}Ly6C^{+}$ cells in the hippocampus. (f) Representative FACS plots showing NOS-2 expression gated on $CD45l^{ow/-}CD11b^{+}$ microglia cells. Green filled histogram represents isotype control. Cumulative data showing the percentages of $CD45\text{-}CD11b^{+}NOS\text{-}2^{+}$ (M1-like) microglia. (g) Representative FACS plots showing Arginase-1 expression gated on $CD45l^{ow/-}CD11b_{+}$ cells. Green filled histogram represents isotype control. Cumulative data showing the percentages of $CD45^{-}CD11b^{+}Arginase\text{-}1^{+}$ (M2-like) microglia. Results are presented as mean ± SEM and are obtained from n = 4-5 mice /group, from 3 independent experiments. Statistical significant was obtained by unpaired Student t-test and Mann Whitney t test when normal distribution was not valid (Control vs 4 days DSS, Control vs 7 days DSS, Control vs 2 cycles DSS, ***P<0.001, **P<0.01, *P<0.05) (4 days DSS vs 7 days DSS, 4 days DSS vs 2 cycles DSS, $^{###}P<0.001$, $^{##}P<0.01$, $^{#}P<0.05$) (7 days DSS vs 2 cycles DSS, $^{\&\&\&}P<0.001$, $^{\&\&}P<0.01$, $^{\&}P<0.05$).*

3.11 Distinct cytokine profiles in the hippocampus over the course of colitis

Cytokines are considered strong modulators of neuronal activity [38] and hippocampal neurogenesis(321) and control intricate interactions between innate immune cells in the periphery and the brain(328).

Significant increases in the circulating levels of the pro-inflammatory cytokines TNF-α (***P*=0.0043 and **P*=0.0357) and IL-1β (***P*=0.0043 and **P*=0.0159) were detected on day 4 and 7 of acute colitis, with those of IL-6 peaking on day 7 (****P*<0.0001) (**Figure 16 a**). Notably, levels of all three cytokines were elevated in the hippocampi of mice with acute colitis, compared to control mice [TNF-α (**P*=0.0155 and ****P*<0.0001), IL-1β (***P*=0.0070 and ***P<0.0001) and IL-6 (**P*=0.0194 and ****P*=0.0005)] (**Figure 16 b**). Interestingly, the levels of the anti-inflammatory cytokine IL-10 were steadily elevated both in the serum (****P*<0.0001) and hippocampus (**P*=0.0454 and ****P*<0.0001) during acute DSS colitis.

In contrast, and in line with the microglia immunophenotypes described above, the levels of TNF-α (serum:$^{\#\#}P$=0.0080 and $^{\&}P$=0.0358; hippocampus: $^{\&}P$=0.0290), IL-1β (serum: $^{\#\#}P$=0.0095 and $^{\&}P$=0.0286; hippocampus: $^{\&}P$=0.0104) and IL-6 (serum: $^{\#}P$=0.0250 and $^{\&}P$=0.0331; hippocampus: $^{\&\&}P$=0.0033) were decreased in chronic disease compared to acute colitis, both in the circulation and the hippocampus (Figure 6 b), while the levels of the anti-inflammatory cytokine IL-10 (serum: ***P<0.0001; hippocampus: ***P<0.0001) remained higher compared to control mice (**Figure 16 a and b**).

These pro- and anti –inflammatory cytokines profiles closely mirror the changes observed in the phenotype of microglia cells during the distinct phases of acute and chronic colitis.

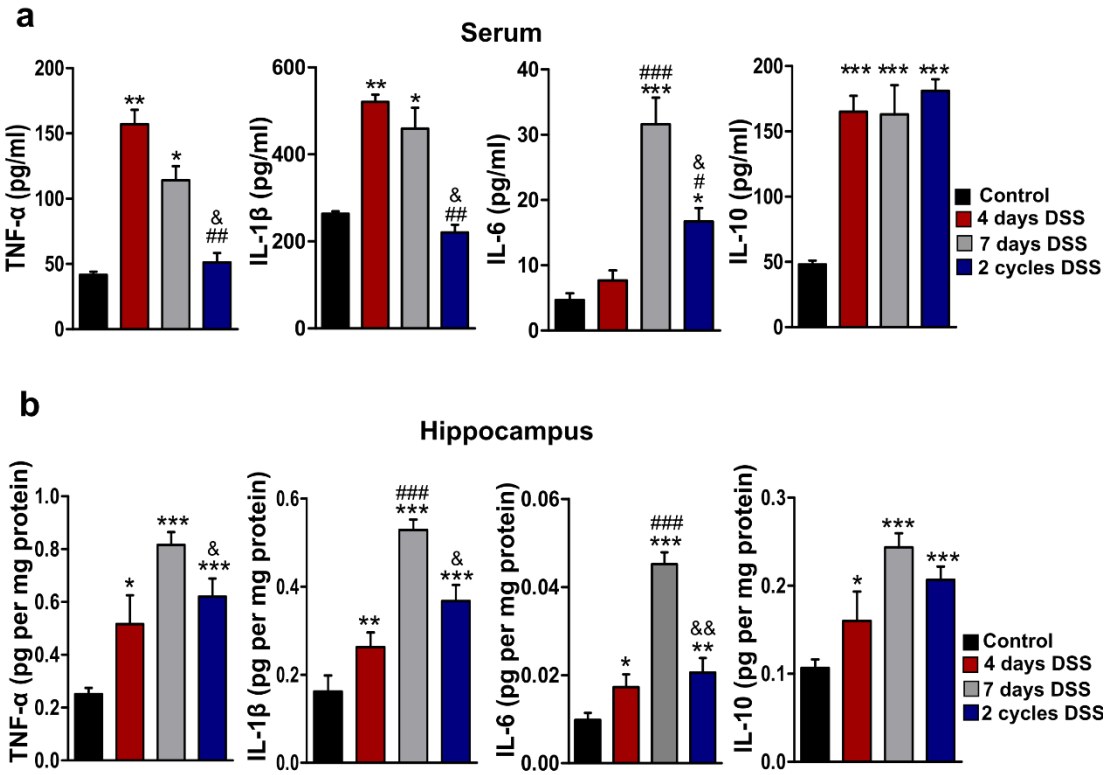

Figure 16. Cytokine profile in the brain and the periphery during acute and chronic DSS colitis. (a) TNF-α, IL-1β, IL-6 and IL-10 release was measured by ELISA in the sera and hippocampal homogenates of control and DSS-treated mice, as indicated. Results are presented as mean ± SEM and are obtained from n = 4-5 mice /group, from 2 independent experiments. Statistical significant was obtained by unpaired Student t-test and Mann Whitney t test when normal distribution was not valid (Control vs 4 days DSS, Control vs 7 days DSS, Control vs 2 cycles DSS, ***P<0.001, **P<0.01, *P<0.05) (4 days DSS vs 7 days DSS, 4 days DSS vs 2 cycles DSS, $^{###}$P<0.001, $^{##}$P<0.01, $^{#}$P<0.05) (7 days DSS vs 2 cycles DSS, $^{\&\&\&}$P<0.001, $^{\&\&}$P<0.01, $^{\&}$P<0.05).

3.12 Microglia activation in the neurogenic niche of the hippocampus during acute and chronic DSS colitis

Considerable evidence suggest that microglia modulate adult neurogenesis in the hippocampus(255,256). Specifically, microglia participate in stem cell proliferation and survival through their release of important trophic factors and anti-inflammatory cytokines(276).

Given their role in the regulation of hippocampal neurogenesis we next assessed their activation profile during acute and chronic DSS colitis in the neurogenic niche of the hippocampus via immunohistochemical and morphological analysis. Notably, the pattern of the aforementioned increase in the microglial cells of the hippocampus was also evident in the microglia residing in the neurogenic niche (SGZ) of the DG in mice with acute DSS colitis compared to control, as assessed by Iba-1 staining (Control: 818.2±41.69 vs 4 days DSS: 1302±103.7 cells/mm^3, *P*<0.001; Control: 818.2±41.69 vs 7 days DSS: 1092±68.07 cells/mm^3, *P*<0.01; **Figure 17 a and b**). Moreover, increased numbers of Iba-1$^+$ cells in the neurogenic niche of the DG of mice were maintained in chronic colitis (Control: 822.9±69.57 vs 2 cycles DSS: 1080±54.73 cells/mm^3, *P*<0.05; Control: 822.9±69.57 vs 3 cycles DSS: 1179±72.56 cells/mm^3, *P*<0.05; **Figure 17 c and d**). This increase in microglia was also evident in the granule cell layer (GCL) of the DG early on during acute DSS as well as in the chronic phase (Control: 979±98.82 vs 4 days DSS: 1965±132.4 cells/mm^3, *P*<0.001; Control: 1416±67.63 vs 3 cycles DSS: 1992±135.2 cells/mm^3, *P*<0.05; **Figure 17 a, b, c and d**). Surprisingly, no differences were observed in the number of microglia cells in the hilus of the DG during acute and chronic DSS colitis (*P*>0.05; **Figure 17 a, b, c and d**). These data indicate that microglia are increased and actively integrated in the neurogenic niche of the hippocampus during acute and chronic DSS colitis.

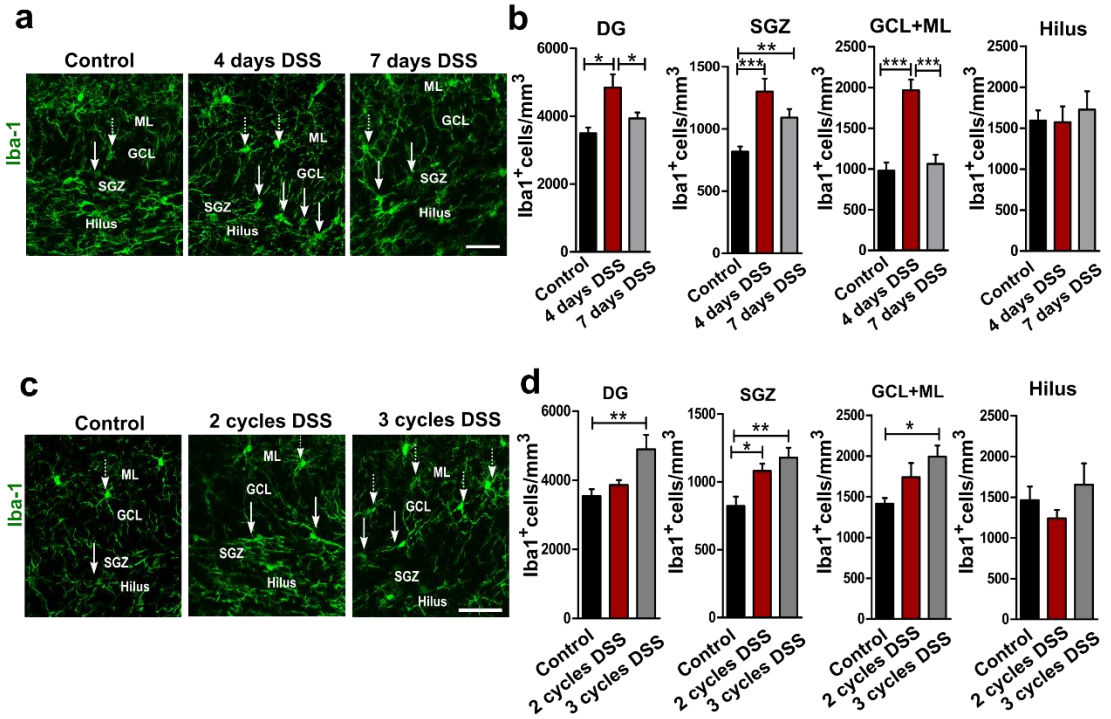

Figure 17. Effects of acute and chronic DSS colitis on microglia in the DG. (a) *Representative confocal maximal projection images of Iba-1+ cells in the various sub-regions of the DG in control and mice with acute DSS colitis (4 and 7 days). Arrows depict Iba-1+ cells in the SGZ, GCL and ML sub-region. Scale bar: 50 µm. (b) Stereological quantification of Iba-1 + cells in the sub-regions of the DG (n=5-12) (c) Representative confocal maximal projection images of Iba-1+ cells in the various sub-regions of the DG in control and mice with chronic DSS colitis (2 and 3 cycles DSS). Arrows depict Iba-1+ cells in the SGZ, GCL and ML sub-region. Scale bar: 50 µm. (d) Stereological quantification of Iba-1 + cells in the sub-regions of the DG (n=6-9). Each value represents the mean±SEM. One way ANOVA with Tukey's post hoc test (***P<0.001; **P<0.01; *P<0.05). DG: Dentate Gyrus, SGZ: Subgranular zone, GCL: Granule Cell Layer, ML: Molecular Layer*

To further characterize the response of the microglia within the neurogenic niche in acute colitis, we assessed for its polarization to the classical inflammatory phenotype (M1). As described, M1 microglia cells undergo characteristic morphological changes such as swelling of the body, retraction of the processes and decrease of their overall length, adopting a characteristic amoeboid shape. Sholl analysis of individual microglia cells of the DG early on during acute DSS colitis revealed increased soma area (P=0.0001; **Figure 18 a, b and c**), decreased number of primary branches (P=0.0004; **Figure 18 c**) and decreased maximum process (P<0.0001; **Figure 18 c**). These results are in line with the previous findings suggesting that early DSS colitis induces the activation of microglia toward the classical inflammatory phenotype (M1). On the contrary, Sholl analysis in microglia cells of the DG in mice with 7 days of DSS colitis did not detect any cells with this particular phenotype (**Figure 18 d**). These results are also in line with the previous findings showing that most of microglia cells in the hippocampus of mice with 7 days of DSS colitis acquire the M2 phenotype.

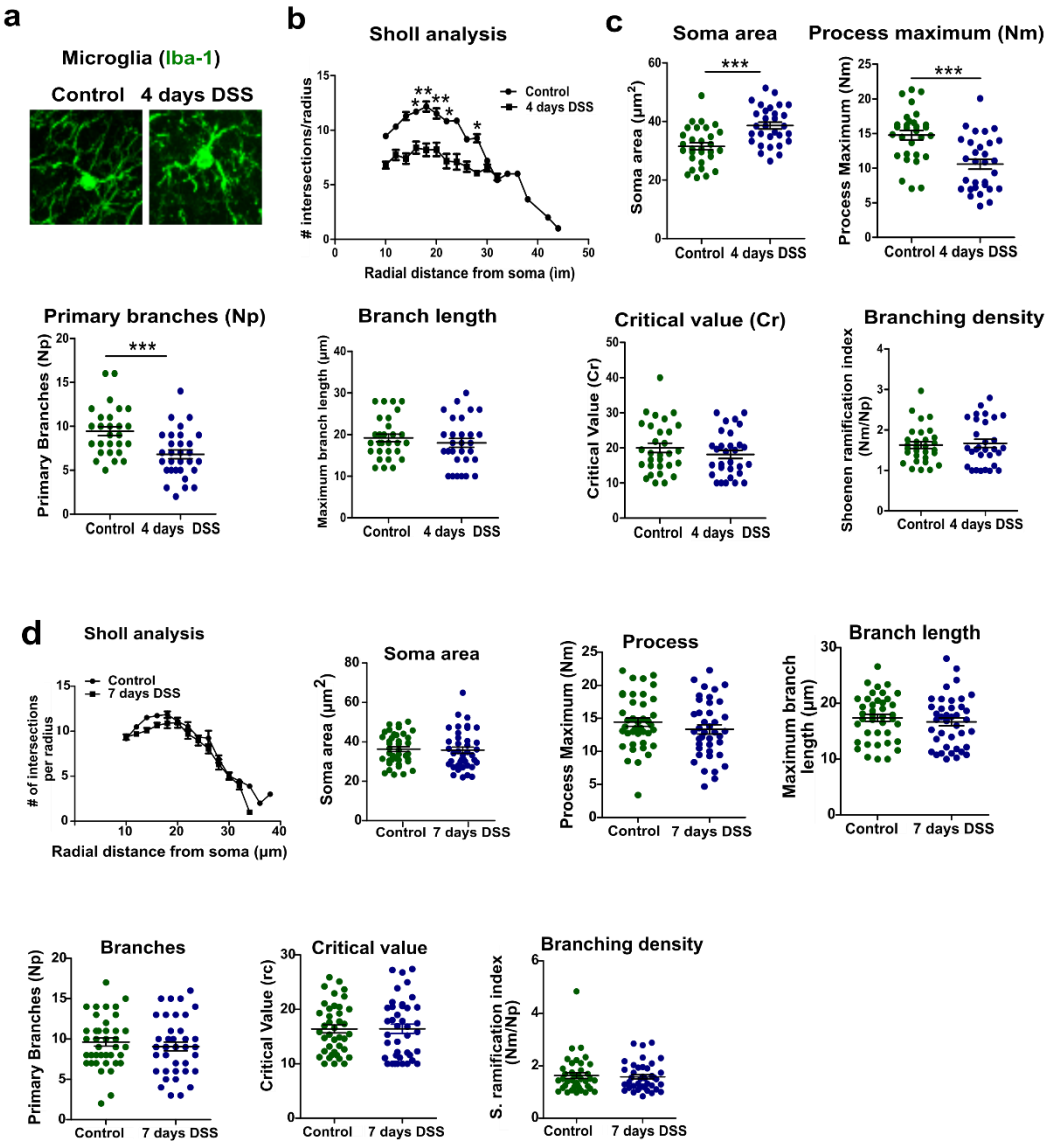

Figure 18. Acute DSS colitis effects on DG microglia morphology. *(a) Representative images of individual DG microglia from control and mice with 4 days of DSS colitis. (b,c,d) Sholl analysis of individual DG microglia cells (n=40 cells/group, n=4). Each value represents the mean±SEM. Student unpaired t-test (***P<0.001).*

Moreover, Sholl analysis revealed morphological alterations in DG microglia of mice with chronic DSS colitis (3 cycles), including increases in the soma area (*P*=0.0029; **Figure 19 a and b**), the number of processes (*P*=0.0423; **Figure 19 b**), in the distance from the soma to the end of the maximum process (*P*=0.0009; **Figure 19 b**) as well as the density of the branching cells (*P*=0.0209**; Figure 19 b**). This phenotype is a hallmark of the M2 polarization state, characteristic of alternatively activated microglia associated with transition from acute to chronic inflammatory feature.

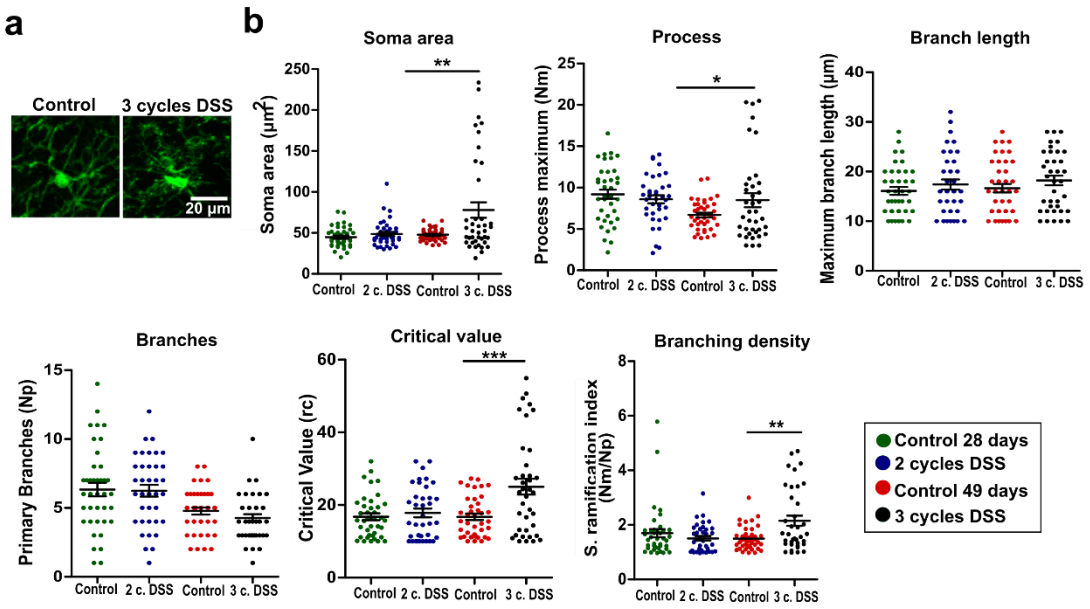

Figure 19. Chronic DSS colitis effects on DG microglia morphology. *(a) Representative images of individual DG microglia from control and mice with chronic DSS colitis. (b) Sholl analysis of individual DG microglia cells (n=40 cells/group, n=4). Each value represents the mean±SEM. Unpaired t-test (****P<0.001, **P<0.01, *P<0.05).*

3.13 Acute DSS colitis induces reactive astrogliosis which is abolished in the Chronic DSS colitis

Astrocytes have been shown to exert a supportive role in neuronal function as well as in NSCs in adult hippocampus(315). Further, astrocytes are considered as cells exhibiting immune-like responses(285), as they have been shown to get activated by inflammatory mediators to counteract the effects of activated microglia. The specific marker for activated astrocytes we used is the cytoskeletal Glial fibrillary acidic protein (GFAP). As shown, GFAP protein levels were significantly increased in the hippocampus of mice with acute DSS colitis (P=0.0180; **Figure 20 a and b**), whereas they were back to control levels in chronic DSS colitis (P > 0.05; **Figure 20 a, c and d**). We next assessed for potential activation of the astrocytes in the DG specifically, as they are key for the neurogenic niche and have been reported to contribute both in the proliferation of NSCs and the synaptic integration of newly formed neurons. Acute DSS colitis induced a profound increase of $GFAP^+$ cells in the DG (P<0.01; **Figure 20 e and g and Table 2**).

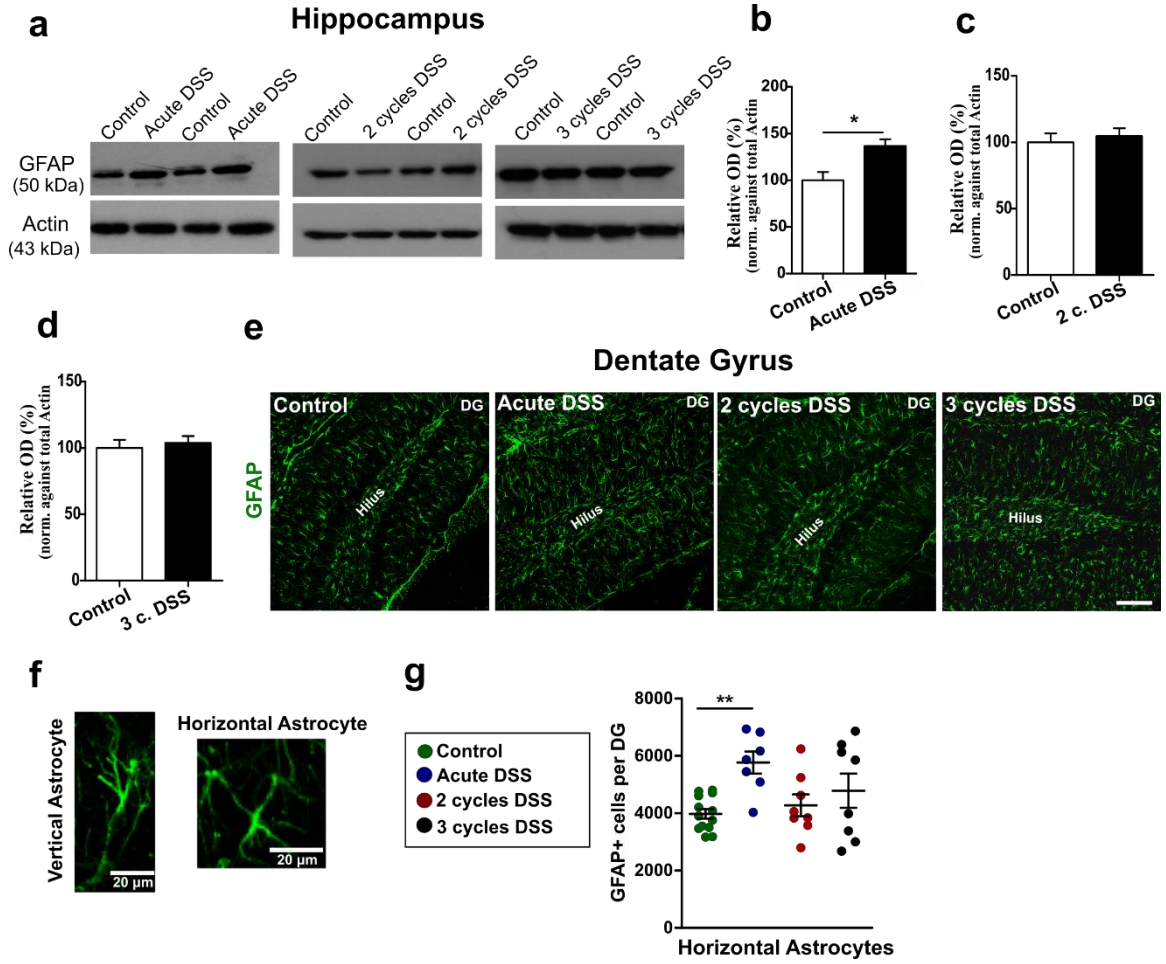

Figure 20. Induction of hippocampal astrocytosis in acute DSS colitis. (*a) GFAP expression in the hippocampus of control and mice with acute and chronic DSS colitis. (b,c,d) Relative protein expression of GFAP in the hippocampus of control and mice with acute and chronic DSS colitis (n=4-5). Student unpaired t-test. *P<.05. (e) Representative confocal maximal projection images of GFAP+ cells in the DG of control and mice with acute and chronic DSS colitis. Scale bar: 100 µm (f) Representative images of individual DG vertical and horizontal astrocytes. (g) Stereological quantification of GFAP $^{+}$ cells in the DG control and mice with acute and chronic DSS colitis. One way ANOVA with Turkey post hoc test (**P<.01; n=8-13).*

There are two distinctive types of astrocytes in the DG, the "vertical" and the "horizontal", the later referring to non -proliferative cells implicated in immune processes(315), abundant in the hilus, the granule and the molecular cell layers (**Figure 20 f**). Morphological analysis of the horizontal type (**Figure 21 a, b**) revealed minor morphological changes and a decrease in the branch length (*P*=.0411; **Figure 21 b**) in mice with acute colitis. Moreover, a small proportion of astrocytes within the hilus exhibited increased soma area, a morphological characteristic of reactive astrocytosis (**Figure 21 c**). These reactive astrocytosis-characteristic findings seem to constitute a transient response, as they were not present in chronic DSS colitis.

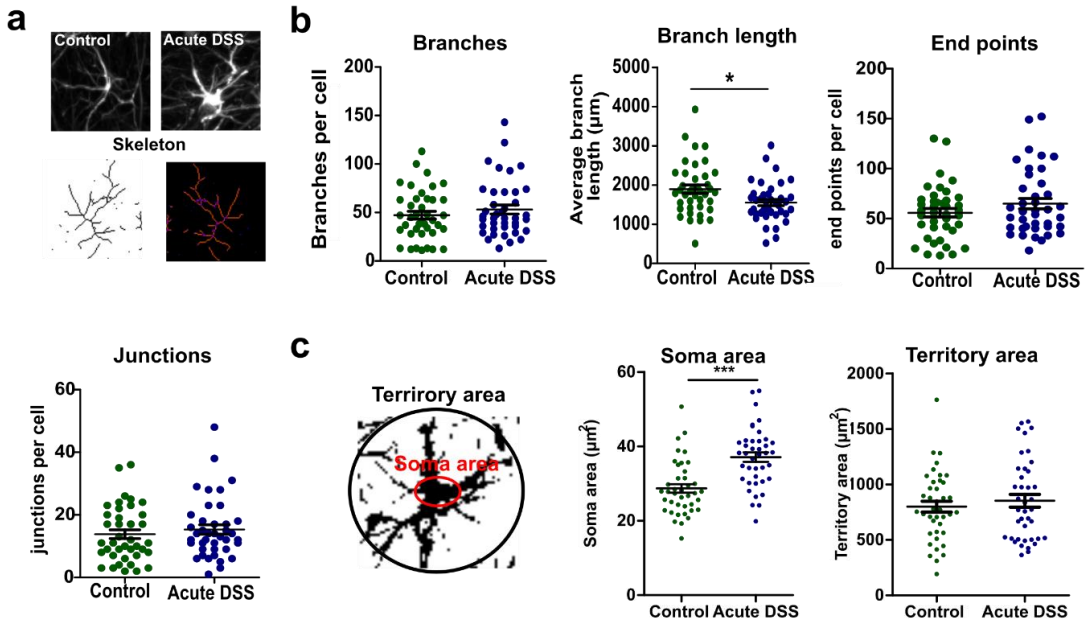

Figure 21. Acute DSS colitis did not induce morphological alterations in DG astrocytes. (*a,b) Skeleton analysis of individual DG horizontal astrocytes in control and mice with acute DSS colitis (n= 40 cells/group, n=4). Student unpaired t-test. *P<.05. 2 independent experiments. (c) Binary image of a horizontal astrocyte. Schematic representation of the way we calculate the soma area (red cycle) and the territory area (black cycle) of an individual astrocyte using ImageJ. Quantification of Soma area (µm2) and Territory area (µm2) (n= 40 cells/group, n=4). Each value represents the mean±SEM. Student unpaired t-test. *P<.05.*

3.14 Acute DSS colitis decreases the activity of the GSK3β kinase in the hippocampus which is normalized in chronic DSS colitis

GSK3β has been associated with microglia migration, inflammatory responses and neurotoxicity through activation of astrocytes, whereas inhibition of GSK3β was found to have anti-inflammatory effects (329) in the CNS and peripheral sites. To get a better understanding of the activation status of microglia and astrocytes in the hippocampus of mice with acute and chronic DSS colitis, we evaluated the protein levels of ^{pSer9}GSK3β, as a potential marker for the mediating mechanisms in this disease(329). Indeed, acute DSS colitis induced a profound increase in the protein levels of ^{pSer9}GSK3β in the hippocampus (*P*=0.0343; **Figure 22 a and b**) which was abolished in the chronic disease (*P*>0.05; **Figure 22 c and d**) that, interestingly showed decreased total GSK3β protein levels (*P*<0.05; **Figure 22 c and d**). Increased expression of Arginase-1 (*P*=0.0379; **Figure 22 e**), a marker for anti-inflammatory macrophages, lack of activation of iNOS (*P*>0.05; **Figure 22 f**), a relevant pro-inflammatory mediator and elevated levels of the anti-inflammatory cytokine IL-10 in the hippocampus of mice with acute colitis is in line with these findings(243).

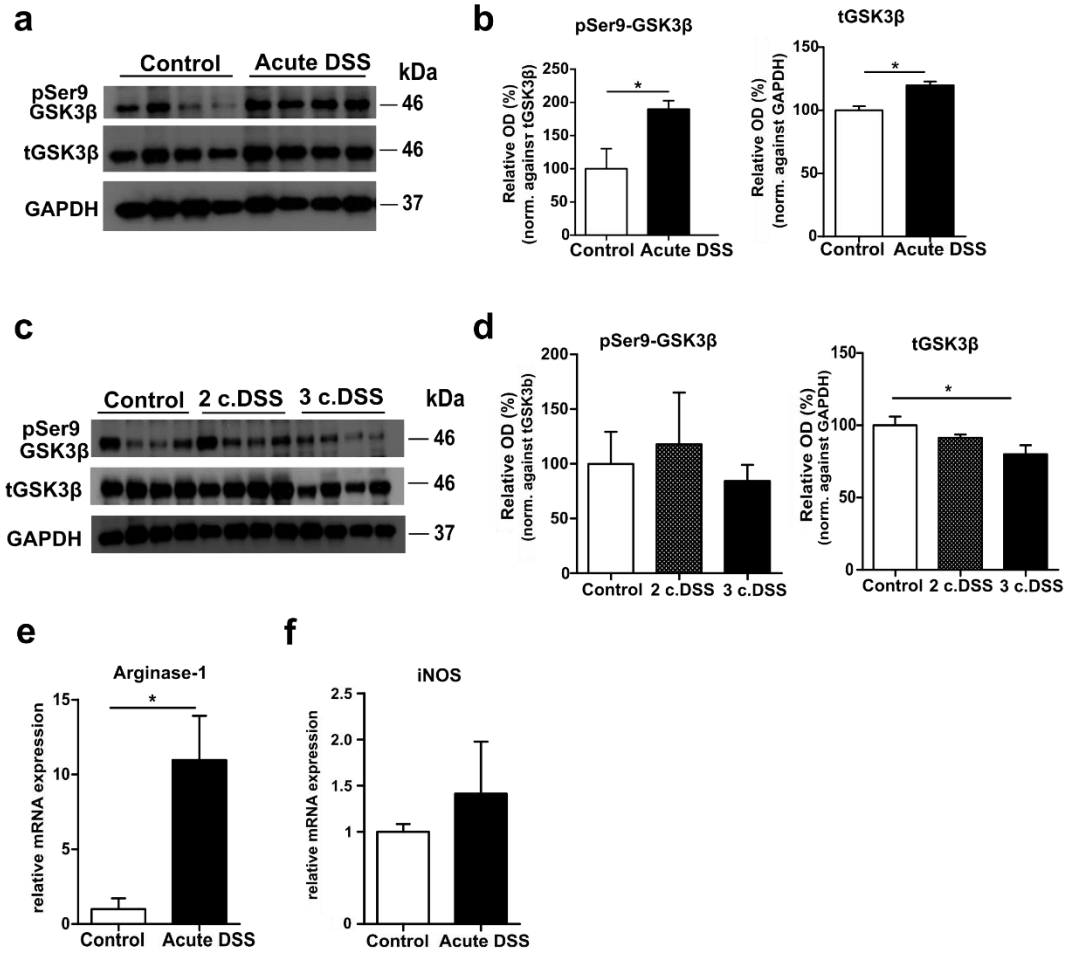

Figure 22. Acute DSS colitis inhibits GSK3β activity in the hippocampus. *(a) ^{pser}9GSK3β and total GSK3β (tGSK3β) expression in the hippocampus of control and mice with acute DSS colitis. (b) Relative expression of ^{pser9}GSK3β and total GSK3β (tGSK3β) expression in the hippocampus (n=4-5). Student unpaired t-test.*P<0.05. (c) ^{pser9}GSK3β and total GSK3β (tGSK3β) expression in the hippocampus of control and mice with chronic DSS colitis. (d) Relative expression of ^{pser9}GSK3β and total GSK3β (tGSK3β) expression in the hippocampus of control and mice with chronic DSS colitis (n=4). One way ANOVA with Turkey post hoc tests. P>0.05, unequal variances analysed with Kruskal Wallis test followed by Dunn's Multiple Comparison Test. *P<0.05 (e,f) Relative mRNA expression of Arginase-1 and iNOS gene in the hippocampus of control and mice with acute DSS colitis. (n=4-5). Student unpaired t-test. *P<0.05. Each value represents the mean±SEM.*

3.15 Acute and Chronic DSS colitis differentially regulates the activation of PI3K/mTOR molecular pathway in the hippocampus.

Activation of glia cells is known to induce several intracellular signaling pathways such as the PI3K/AKT pathway which regulates inflammatory responses, cellular activation and apoptosis(330). The activation of this pathway triggers a signaling cascade which leads to NF-kB translocation. PI3K activation leads to the activation of mTOR- an atypical serine/ threonine protein kinase belonging to the PI3K related kinase family(330).

Phosphorylation of mTOR is known to activate microglia and plays an important role in the regulation of NF-kB activity and inflammation(331).

To this extend we evaluated the protein expression levels of the PI3K kinase, mTOR and NF-kB in the hippocampus of control and mice with Acute and Chronic DSS colitis. PI3K kinase protein levels were upregulated in the hippocampus of mice with Acute DSS colitis (*P*<0.05; **Figure 23 a and b**) whereas in mice with chronic DSS colitis were downregulated (*P*<0.05; **Figure 23 a and c**).

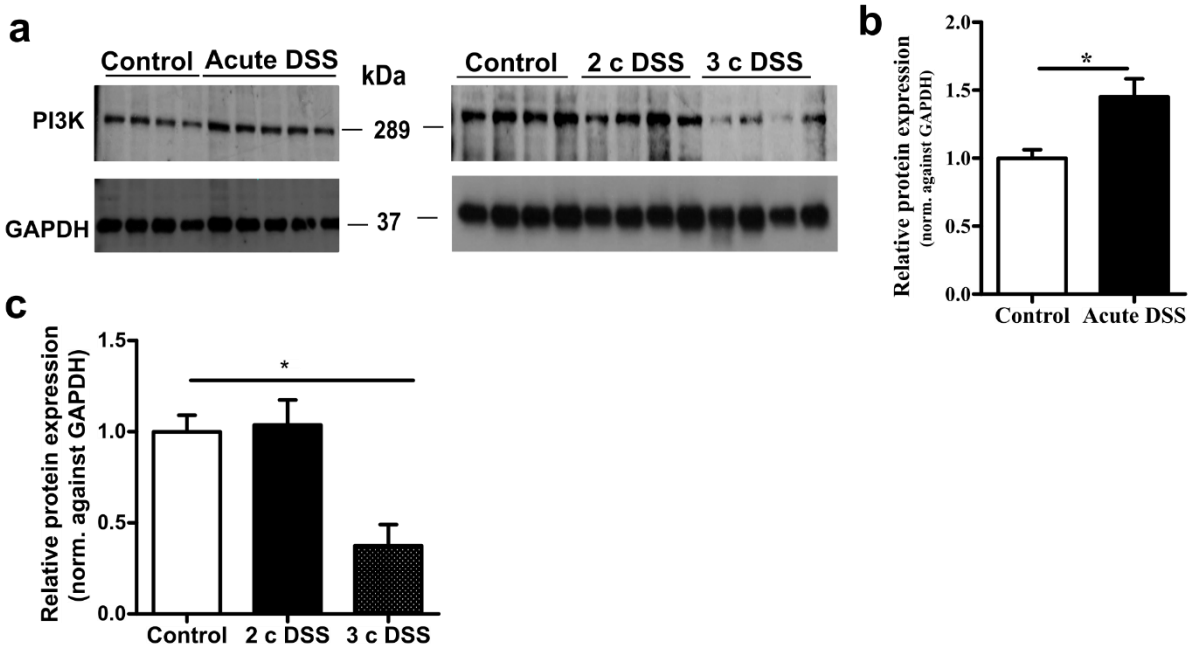

Figure 23. Acute DSS colitis enhances the protein levels of PI3K kinase in the hippocampus. *(a) PI3K expression in the hippocampus of control and mice with acute and chronic DSS colitis. (b) Relative expression of PI3K expression in the hippocampus of control and mice with acute DSS colitis (n=4-5). Student unpaired t-test. *P<0.05. (c) Relative expression of PI3K in the hippocampus of control and mice with chronic DSS colitis (n=4). One way ANOVA with Turkey post hoc tests. *P<0.05. Each value represents the mean±SEM.*

The protein levels of mTOR were also upregulated in the hippocampus of mice with acute DSS colitis (*P*<0.05; **Figure 24 a and b**) which were normalized in the hippocampus of mice with chronic DSS colitis (*P*>0.05; **Figure 24 d and e**). The increase in the protein levels of mTOR were not accompanied with increased protein levels of phosphorylated mTOR in the hippocampus of mice with acute DSS colitis (*P*>0.05; **Figure 24 a and c**). The protein levels of NF-kB were unaltered both in mice with acute and chronic DSS colitis compared to control (*P*>0.05; **Figure 25 f-i**). These data further indicate the anti-inflammatory response of the hippocampus in acute and chronic intestinal inflammation.

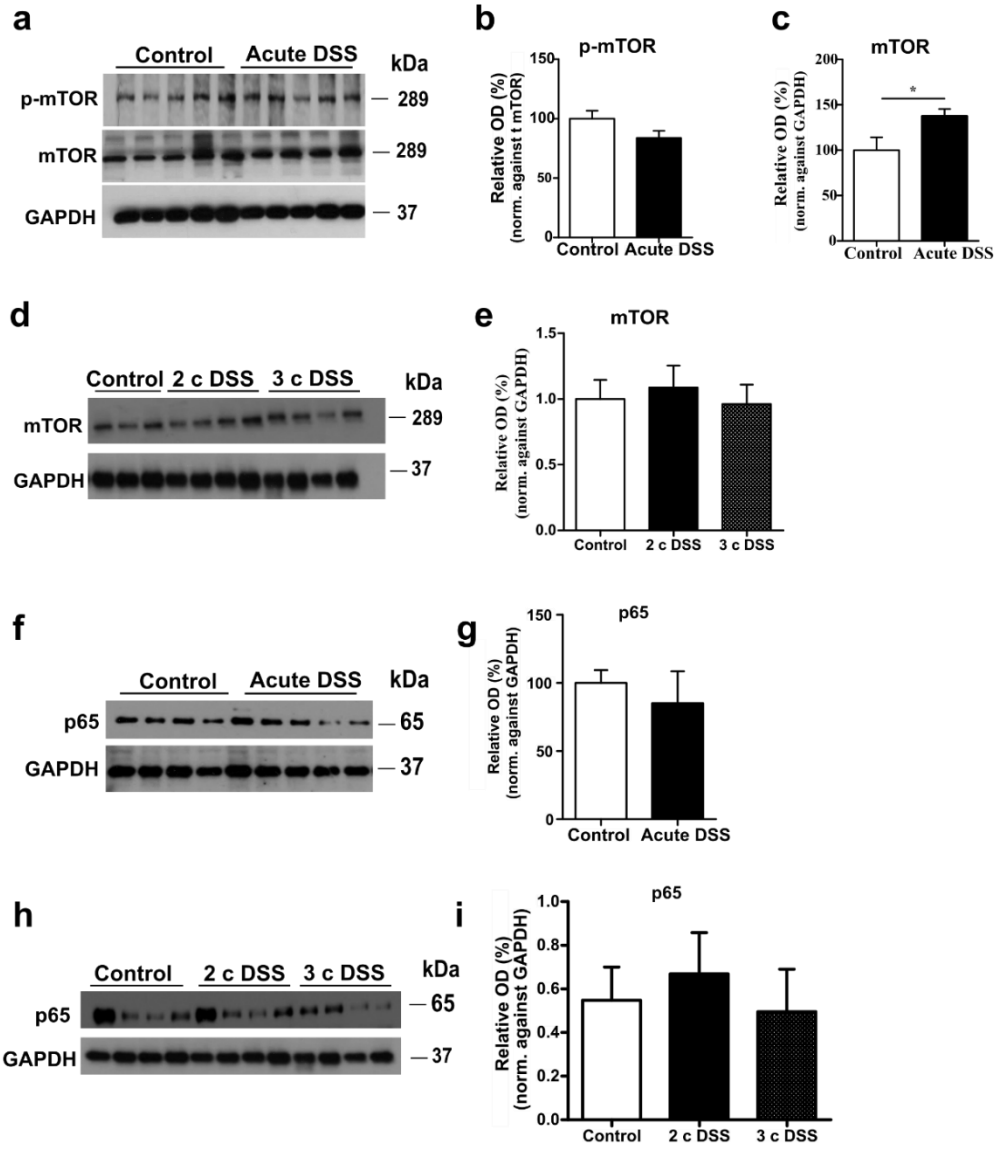

Figure 24. Acute DSS colitis induces upregulation of hippocampal mTOR protein levels. *(a) phospho-mTOR and mTOR expression in the hippocampus of control and mice with acute DSS colitis. (b,c) Relative expression of phospho-mTOR and mTOR in the hippocampus of control and mice with acute DSS colitis (n=4-5). Student unpaired t-test. *P<0.05. (d) mTOR expression in the hippocampus of control and mice with chronic DSS colitis. (e) Relative expression of mTOR in the hippocampus of control and mice with chronic DSS colitis (n=4). One way ANOVA with Turkey post hoc tests. P>0.05. (f) p65-NFkB expression in the hippocampus of control and mice with acute DSS colitis. (g) Relative expression of p65-NFkB in the hippocampus of control and mice with acute DSS colitis (n=4-5). Student unpaired t-test. P>0.05. (h) p65-NFkB expression in the hippocampus of control and mice with chronic DSS colitis. (i) Relative expression of p65-NFkB in the hippocampus of control and mice with chronic DSS colitis (n=4). One way ANOVA with Turkey post hoc tests. P>0.05. Each value represents the mean±SEM.*

3.15 Regulation of *BDNF* expression during acute and chronic DSS colitis

Neurotrophins play a significant role in the maintenance and survival of the peripheral and central nervous systems and mediate several forms of synaptic plasticity(332). Neurotrophins include the nerve growth factor (NGF), the brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), neurotrophin 4/5 (NT4/5) and glial-derived nerve factor (GDNF). Neurotrophic factors interact with two distinct receptors: the tropomyosin receptor kinase (TrK) which belongs to the family of receptor tyrosine kinases and the common p75 neurotrophin receptor (p75NTR) which belongs to the tumor necrosis factor receptor (TNFR) superfamily of death receptors(332). Three different Trks have been identified in mammals: TrkA, TrkB and TrkC. NGF is the preferred ligand of TrkA, BDNF for TrkB and NT3 for TrkC. While these Trk receptors bind only mature neurotrophins, p75NTR interacts with mature neurotrophins as well as with pro-neurotrophins increasing the complexity of the signaling. Among the most studied neurotrophin, the brain derived neurotrophic factor-BDNF is well known to regulate adult hippocampal neurogenesis, synaptic plasticity and is implicated in the progression of neuroinflammation through its ability to induce and being induced by NF-κB(332).

Given the extensive role of BDNF and its receptors on neurogenesis and inflammation we proceeded to the analysis of the levels of BDNF mRNA in the hippocampus of mice with acute DSS colitis. Acute DSS colitis induced an increase in BDNF mRNA levels in the whole hippocampus ($P<0.05$; **Figure 25 a**). However, no detectable differences were found in relative mRNA expression of NGF, TrkB, TrkA, p75NTR in the hippocampus of control and mice with acute DSS colitis ($P>0.05$; **Figure 25 a**). In mice with chronic DSS colitis, the transcriptional levels of BDNF in the hippocampus ($P>0.05$; **Figure 25 b**) were not different compared to control *(*$P>0.05$; **Figure 25 b**).

The mRNA levels of the NGF and the receptors TrkB, TrkA, p75NTR remained unaltered during chronic DSS colitis compared to control ($P>0.05$; **Figure 25 b**). These data indicate that acute colitis transiently increase the gene expression of BDNF in the hippocampus, probably to counteract the effects of inflammation on synaptic plasticity and survival of NSCs/progenitors.

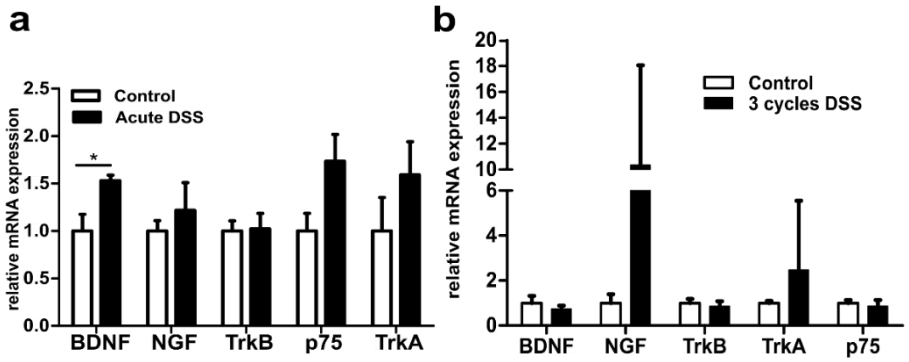

Figure 25. Acute DSS colitis induces the gene expression of BDNF in the hippocampus. *(a) Relative mRNA expression of BDNF, NGF, TrkB, p75 and TrkA genes in the hippocampus of control and mice with acute DSS colitis. (b) Relative mRNA expression of BDNF, NGF, TrkB, p75 and TrkA genes in the hippocampus of control and mice with chronicDSS colitis. Each value represents the mean±SEM. Student unpaired t-test. *P<0.05 (n=4-5 mice/group).*

Tables

Table 1. Acute DSS colitis-Adult hippocampal neurogenesis.

Immunohistochemical markers	Control (mean±SEM) Cells/mm^3	Acute DSS (mean±SEM) Cells/mm^3	Statistical test Unpaired t-test	P value	N
BrdU	1048±75.97	982.5±73.57	t(14)=0.6170	0.547	8
Ki67	1375±103.6	1929±132.9	t(18)=3.291	0.0046	10
Cell cycle exit*	0.5287±0.0461	0.7162±0.01785	t(7)= 3.787	0.0068	7-8
Cell cycle re-enter *	0.4865±0.04723	0.2808±0.01765	t(7)=3.22	0.0074	7-8
GFAP/BrdU	87.30 ± 6.372	133.5 ± 8.312	t(12)=4.407	0.0009	7
GFAP/Sox2/BrdU*	48.68 ± 5.812	92.85 ± 13.38	t(9)= 3.028	0.0143	7-8
GFAP/Ki67*	69.13 ± 5.531	120.1 ± 14.89	t(8)=3.212	0.0124	8
Sox2	3825± 219.8	5490± 386.8	t(11)=3.743	0.0033	8
Sox2/BrdU	485.2 ± 36.62	309.6 ± 37.46	t (13)= 3.341	0.0053	7-8
Sox2/DCX/BrdU*	84.01 ± 7.374	127.7 ± 16.52	t(9)=2.418	0.0387	8
DCX/BrdU	177.2 ± 19.44	115.3 ± 13.62	t(12)= 2.609	0.0228	7
DCX	6011 ± 553.8	8249 ± 494.0	t(18)=3.016	0.0074	10
Caspase 3	182.4 ± 20.92	414.9 ± 33.52	t(10)=5.308	0.0003	5-7
Caspase 3/DCX	19.20 ± 4.800	42.86 ± 6.857	t(10)=2.589	0.0270	5-7

Table 2. Remission phase-Adult hippocampal neurogenesis.

Immunohistochemical markers	Control (mean±SEM)	R. phase (mean±SEM)	Statistical test Unpaired t-test	P value	N
Ki67	1136 ± 104.8	946.8 ± 203.9	t(5)=0.8270	0.4459	5
Sox2	4770 ± 595.9	4224 ± 381.8	t(6)= 0.7714	0.4697	4
DCX	5356 ± 414.4	4932 ± 593.2	t(12)=0.5860	0.5687	6-9
BrdU (13 days p.i)	1809 ± 214.2	2208 ± 68.41	t(6)= 1.774	0.1264	4
BrdU/NeuN (13 days p.i)	312.3 ± 29.46	340.5 ± 30.67	t(6)= 0.6644	0.5311	4
BrdU/GFAP (astrocytic morphology) (13 days p.i)	27.11 ± 9.412	150.6 ± 18.21	t(6)= 6.025	0.0009	4
BrdU/GFAP (activated RGLs) (13 days p.i)	53.54 ± 8.691	71.51 ± 16.18	t(6)= 0.9786	0.3656	4

Table 3. Chronic DSS colitis-Adult hippocampal neurogenesis.

Immunohisto-chemical Markers	Control 28 d. pi (mean± SEM) Cells/mm^3	2 cycles DSS (mean± SEM) Cells/mm^3	Control 49 d. pi (mean± SEM) Cells/mm^3	3 cycles DSS (mean± SEM) Cells/mm^3	Statistical test One way ANOVA	Post hoc test and p-value	N
BrdU	680.3± 57.30	697.7± 71.89	538.3± 64.59	615± 42.65	*P*=0.2543 F(4)=1.439 R^2=01424	Tukey's multiple comparison test Control 28 d pi vs 2 cycles DSS *P*>0.05 Control 49 d pi vs 3 cycles DSS *P*>0.05	7-8
BrdU/NeuN	457.5± 50.27	430.3± 29.68	255.4± 38.19	267± 25.13	*P*=0.006 F(4)=8.032 R^2=0.4810	Tukey's multiple comparison test Control 28 d pi vs 2 cycles DSS *P*>0.05 Control 49 d pi vs 3 cycles DSS *P*>0.05	7-8
BrdU/DCX/ NeuN	26.40 ± 7.960	43.20 ± 8.139	36.00 ± 10.95	26.40 ± 8.818	*P*=0.4702 F(4)=0.8871 R^2=0.1507	Tukey's multiple comparison test Control 28 d pi vs 2 cycles DSS *P*>0.05 Control 49 d pi vs 3 cycles DSS *P*>0.05	4-6

Table 4. Effects of acute and chronic DSS colitis on DG glial populations.

Immunohistochemical Markers	Control (mean± SEM) Cells/mm^3	4 days DSS (mean± SEM) Cells/mm^3	7 days DSS (mean± SEM) Cells/mm^3	Statistical test One way ANOVA	Post hoc test and p-value	N
Iba-1 DG	3496± 173.9	4849±386.3	3939± 174.3	*P*=0.0022 F(3)=8.207 R^2=0.4273	Tukey's multiple comparison test Control vs 4 days DSS ***P*<0.01 Control vs 7 days DSS *P*>0.05	5-12
Iba-1 SGZ	818.2±41.69	1302±103.7	1092±68.07	*P*=0.0001 F(3)=13.97 R^2=0.5708	Tukey's multiple comparison test Control vs 4 days DSS ****P*<0.001 Control vs 7 days DSS ***P*<0.01	5-12
Iba-1 GCL+ML	979±98.82	1965±132.4	1062±111.1	*P*<0.0001 F(3)=17.31 R^2=0.6115	Tukey's multiple comparison test Control vs 4 days DSS ****P*<0.001 Control vs 7 days DSS *P*>0.05	5-12
Iba-1 Hilus	1593±122.4	1571±195	1727±223	*P*=0.8081 F(3)=0.2151 R^2=0.01918	Tukey's multiple comparison test Control vs 4 days DSS *P*>0.05 Control vs 7 days DSS *P*>0.05	5-12
GFAP DG	3959±179.6	5794±356.4	5767±385.3	*P*<0.0001 F(3)=15.9 R^2=0.5911	Tukey's multiple comparison test Control vs 4 days DSS ****P*<0.001 Control vs 7 days DSS ****P*<0.001	6-12
Immunohistochemical markers	**Control (mean± SEM) Cells/mm^3**	**2 cycles DSS (mean± SEM) Cells/mm^3**	**3 cycles DSS (mean± SEM) Cells/mm^3**	**Statistical test One way ANOVA**	**Post hoc test and p-value**	**N**
Iba-1 DG	3543±199.6	3860±148.6	4899±417.8	*P*=0.0098 F(3)=6.141 R^2=0.4194	Tukey's multiple comparison test Control vs 2 cycles DSS *P*>0.05 Control vs 3 cycles DSS ***P*<0.05	6-9
Iba-1 SGZ	822.9±69.57	1080±54.73	1179±72.56	*P*=0.0040 F(3)=7.787	Tukey's multiple comparison test	6-9

				R^2=0.4781	Control vs 2 cycles DSS *P<0.05 Control vs 3 cycles DSS *P<0.05	
Iba-1 GCL+ML	1416±67.63	1740±174.5	1992±135.2	P=0.0203 Kruskal-Wallis statistic: 7.795	Kruskal-Wallis statistic test Control vs 2 cycles DSS P>0.05 Control vs 3 cycles DSS *P<0.05	6-9
Iba-1 Hilus	1463±168.3	1240±103.6	1653±262.9	***P***=0.3891 F(3)=0.9925 R^2=0.09459	Tukey's multiple comparison test Control vs 2 cycles DSS P>0.05 Control vs 3 cycles DSS P>0.05	6-9
GFAP	3978±166.4	4275±378.7	4787±599.4	P=0.2910 F(3)=1.295 R^2=0.09059	Tukey's multiple comparison test Control vs 2 cycles DSS P>0.05 Control vs 3 cycles DSS P>0.05	8-13

4. Discussion

As both the incidence and prevalence of IBD is growing in the developed countries for the last decade or more and available data provide missing information on associated co-morbidities, the link between psychiatric symptoms with the disease activity remains to be elucidated(4). Epidemiological studies suggest that the prevalence of depression and anxiety is much higher in IBD patients as compared to the general population and correlates positively with the severity of the disease(4). There are few functional magnetic resonance (fMRI) studies that identified structural changes or alterations in the activation of cortical and limbic circuits involved in emotional processing and cognitive function in IBD patients(333,334). These studies indicate that there is a biological basis in the mechanisms that affects the functionality of brain circuits at the cellular and molecular level. The mechanisms by which depression and cognitive dysfunction occur in IBD patients are still unknown but a plethora of new studies suggest that an inflammatory environment in the gut leads to behavioural abnormalities characteristic of depressive and anxiety-like symptoms in a bottom-up manner. So far, most studies implicate the activation of the hypothalamic-pituitary-adrenal (HPA) axis and immune system triggered by intestinal inflammation leading to the subsequent release of glucocorticoids which negatively affect hippocampal function and neurogenesis(95). Animal models of colitis have recently addressed stress-related changes in hormones (such as corticosterone), altered gene expression patterns in limbic structures and neuroinflammation mediated by activated microglia as potentially contributors in behavioural defects and cognitive dysfunction(97,335).

Peripheral and CNS inflammation is increasingly recognized as a critical factor in the pathogenesis of numerous psychiatric illnesses(336). A significant proportion of studies point to the connection of immune dysregulation and psychiatric disorders(336). Numerous experimental models of peripheral and CNS inflammation provide strong support for the role of inflammatory mechanisms in the etiology of depression and anxiety disorders(337). These models suggest that pro-inflammatory mediators directly exert negative effects on neurons, ultimately contributing to the development of psychiatric disorders(337). Cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), as well as reactive oxygen species and reactive nitrogen species, disrupt neurogenesis(262), neuronal excitability(338), synaptic transmission, synaptic plasticity and neuronal survival(339). These effects are mainly mediated by microglia the resident innate immune cells, the major mediators of neuroinflammation(339). These cells are strongly implicated in the pathogenesis of neuropsychiatric disorders and constitute the primary functional mediators of the homeostasis of the neurogenic niches in the adult brain(340). As IBD is a chronic inflammatory disease(323), the short and long-term effects of the inflammatory cues in the glia and neural stem cells of the adult brain should be addressed as factors that potentially affect the behavioural and cognitive outcome.

Chemical induced model of colitis recapitulates key immunological and histopathological features of human IBD and constitute valuable models to study the effects of intestinal inflammation on the brain and behaviour(80,83). In the present study, we provide evidence that acute experimental colitis induced pro-inflammatory effects on innate immune

responses not only in the periphery but also in the hippocampus. We also demonstrate colitis-induced enhancement in hippocampal neurogenesis, however with altered cell cycle kinetics of the proliferating progenitors. Additionally, we show that in the chronic phase of colitis, innate immune cells acquired an anti-inflammatory phenotype, accompanied by deficits in the migration and integration of newborn neurons in the functional circuitry of the hippocampal DG. In this study, we sought to investigate the potential effects of colitis on hippocampal neurogenesis, a process underlying a number of brain functions in adults. Our findings demonstrating effects of intestinal inflammation on hippocampal neurogenesis and local innate immune responses, provide potential mechanistic links to relevant IBD co-morbidities and propose that therapeutic strategies targeting immune responses in IBD may be of benefit for the cognition and behavioural changes associated with this disease.

4.1 Enhanced hippocampal neurogenesis as a survival response to acute inflammatory stress associated with colitis

Our findings showed that acute colitis enhanced neurogenesis in the hippocampal niche. This enhancement was accompanied with increased transcriptional levels of BDNF in the hippocampus that is known to support neurogenesis. Further, we found that proliferating progenitors showed decreased duration of the S-phase, indicative of altered cell cycle kinetics, most evident on Type 2a and Type 3 cells of the hippocampal NSCs lineage. The cell type-specific regulation of cell cycle kinetics could be considered as a compensatory mechanism to acute inflammatory responses, elicited to prevent neuronal apoptosis which was increased in the SGZ of mice with acute DSS colitis and to facilitate the transition to more differentiated neuronal cell types. In fact, the increase in the total numbers of cells of the neurogenic lineage may be attributed to the increased cell cycle progression of the quiescent NSC pool (Type 1), which ultimately gives rise to Type 2a cells ($Sox2^+$), as well as, of the Type 2b cells ($Sox2^+/DCX^+$) which give rise to Type 3 cells (DCX^+). Induction of hippocampal neurogenesis under systemic inflammatory conditions is increasingly recognized as a homeostatic response to the inflammatory stressor(341,342).In line, exposure to a repeated predictable stressor was shown to enhance neurogenesis(343), as repeated challenges are associated with minimal, if any, stress responses. In this context, it was recently reported that repeated exposure to predictable stressors was associated with resilience against acute DSS colitis-induced behavioural changes in mice(344). Moreover, corticosterone levels were elevated on the 7^{th} day post DSS administration(97), a transient effect, that is most likely related to the increase of IL-6(345). Most importantly, as glucocorticoids are negative modulators of hippocampal neurogenesis, HPA axis activation would be expected to decrease neurogenesis(192). However, to our surprise, we found enhanced neurogenesis 7 days post DSS administration. Furthermore, in the chronic DSS colitis model, the numbers of proliferating progenitors, neuroblasts and newborn neurons were not reduced. Our findings do not suggest a direct critical role for glucocorticoids in shaping adult hippocampal neurogenesis during experimental colitis. It is of great interest to note here that suppression of hippocampal neurogenesis leads to activation of HPA axis(346). In mice with disrupted neurogenesis, glucocorticoid levels are slower to recover after moderate stress, and are less suppressed by dexamethasone(346). Our data may indicate a suppressive effect of adult hippocampal neurogenesis in the activation of HPA

axis during acute DSS colitis, but further studies are needed to confirm this hypothesis. As glucocorticoids in pharmacological doses are often administered as part of the therapeutic strategies for inflammatory diseases(347), it would be helpful to assess in these patients their long-term effects on neurogenesis and associated processes. Taken together, our findings suggest that the enhanced survival response in the hippocampal neurogenic niche we describe in the present study, may be developed to counteract the effects of chronic inflammatory stress associated with colitis.

Besides the effects of HPA axis in the progression of a peripheral inflammation, much attention has been given on the role of the vagus nerve in modulating inflammatory responses(12). It is now well-documented the existence of a vagally-mediated anti-inflammatory reflex in which the presence of proinflammatory cytokines in the periphery is detected by vagal afferents, resulting in a vagal efferent response associated with an attenuation of cytokine release from macrophages via nicotinic acetylcholine receptors(12). Moreover, vagotomy can accelerate LPS-induced septic shock and increase systemic TNF production, while selective inhibition of vagal afferent fibers worsens hapten-induce colitis(299). On the contrary, the stimulation of the vagus nerve selectively down-regulates the production of proinflammatory cytokines. Acute stimulation of vagus nerve in rats increases the proliferation of hippocampal NSCs/progenitors apparent 24 h and 3 weeks after treatment. It is of note that it also increases the number of neuroblasts(298). Conversely, vagotomy reduced proliferation and survival of newly born cells as well as decreased the number of neuroblasts and their complex dendritic morphology. These data support the hypothesis that induction of hippocampal neurogenesis during acute colitis may be related to the anti-inflammatory actions of stimulated vagus nerve, due to the release of pro-inflammatory cytokines in the intestine. Further studies are needed to explore this interesting possibility. Moreover, the observed dissociation between the vasculature and proliferating progenitors in the SGZ of the DG in mice with acute DSS colitis may induce long-term defects in maturation, differentiation and migration process of newly born neurons. This dissociation possibly occurs by circulating inflammatory factors that negatively affect the proliferation of NSCs/progenitor cells.

4.2 Normalized levels of hippocampal neurogenesis during Remission phase

The remission phase of colitis is characterized by increased anti-inflammatory cytokines in the periphery, resolution of inflammation in the intestinal system and repair of the epithelium of the gut(323). IBD patients with active symptoms are more likely to present depressive or anxiety behaviour, while in remission the percentage of patients suffering from psychiatric disorders decline(49). Further studies are needed to confirm the link between the active and inactive periods of IBD with psychiatric comorbidities. The resolution of intestinal inflammation occurs in a time-frame of 14 days in the DSS model(83) where the first week after DSS administration is characterized by severe clinical features with a rise of cytokines derived from helper T cells (Th1) in the gut and the systemic circulation(83). Previous studies have shown that inflammation decreases the differentiation and newborn neurons survival in the adult hippocampus(261,262). Interestingly, the number of survived neurons in remission phase was unaltered. This can be attributed to the enhanced production of progenitors and neuroblasts during acute DSS colitis that constitute a reservoir that is able

to negate the potential reduction of newly born neurons during the progress of colitis. In the behavioural level, it has been shown that rats in remission phase after DSS administration exert decreased compulsive behaviour as assessed by marble burying test(92). Usually, increased marble burying indicate neophobia and anxiety(92). As anxiety traits have been considered to be partially neurogenesis-mediated, we can hypothesize that the normal levels of neurogenesis in the hippocampus during the remission phase could partly contribute to the observed effects in the anxiety traits of mice with remission.

It has been previously shown that inflammatory stimuli can switch the cell fate of neural stem cells towards to astrocytes(262,348). Remission phase induced a profound increase in astrogenesis. This increase might be a survival response to the noxious stimuli coming from the periphery to counteract the development of neuroinflammation with debilitating effects on neurogenesis process and the metabolic support of mature neurons in the DG. This might refer to the enhancement of trophic support of neural stem cells and mature neurons to function under "inflammatory stress" as well as to increase the number of astrocytes wrapped around blood vessels in the DG to prevent further leakage of inflammatory mediators.

4.3 Deficits in the migration and integration of newborn neurons in the functional circuitry of the DG might lead in cognitive dysfunctions and mood alterations

Newborn neurons in the hippocampus are implicated in most sophisticated cognitive functions, such as, pattern separation and cognitive flexibility, while they are critical regulators in the firing process of mature granule neurons of the DG through feedback inhibition mechanisms(149). Dysregulated maturation processes of the newborn neurons, including migration and integration, have been implicated in deficiencies in memory and learning, as well as, in abnormal mood behaviors(229,271). We show that chronic DSS colitis induced deficits in the migration patterns of newborn neurons in the functional circuitry of the DG, with debilitating effects on their downstream integration. Indeed, newborn neurons migrated in a short distance inside the GCL of the DG in mice with chronic intestinal inflammation. Previous studies have shown that chemokines regulate the migration of neural precursors to sites of neuroinflammation. In murine models of cranial irradiation or seizures the new neurons have the capacity to migrate to the site of damage in response to the chemokine MCP1/CCR2 signalling pathway(324). However, the mechanisms that mediate the distorted migration pattern of new neurons in the DG during peripheral inflammation are largely unknown and need further evaluation.

We hypothesized that the altered migration pattern in chronic DSS colitis may impact on the establishment of functional connections between the mature dentate granule neurons and the synaptic inputs from sub-regions of hippocampus, such as the CA3 area, and other brain regions involved in cognitive function. It has been proposed that adult hippocampal neurogenesis regulate information processing in the hippocampus, and newborn neurons may contribute to the circuitry both by integrating new information and by inhibiting the activity of mature neurons(149). The inhibitory effect of newborn neurons may be essential to erase previously established for example anxiety-associated memories and to allow new,

non-anxiety-associated memories to be formed instead (cognitive flexibility)(149). This inhibitory effect may facilitate sparse encoding of new information (pattern separation). These functions together may reduce the interference between the previous memory of a stressful context and the experience of a new safe context(149). Alterations in these processes related to an inflamed environment can disrupt the integration of new information and the inhibition of the activity of the dentate granule mature neurons. Indeed, exposure of mice with chronic colitis in a novel spatial environment did not enhance the expression of the immediate early gene *Arc*(325), a marker of neuronal activity, in newborn and mature neurons in the DG. These data suggest that the herein identified abnormal distribution of the newborn neurons impacts on their functional integration into behavior-relevant hippocampal networks. In agreement with our studies, previous reports have documented ectopic distribution and decreased integration of newborn neurons in the DG circuitry in neuro-inflammatory conditions, such as, brain injury(349), stroke and LPS- induced neuroinflammation(229). Although, clinical studies have documented increased incidence of memory loss in IBD patients, attention deficits and declining executive functions(350), it remains unknown whether these deficits are neurogenesis-mediated. A recent study reported task-switching deficits, an indicator of cognitive inflexibility in IBD patients(334). Considering that cognitive flexibility is increasingly recognized as a hippocampal neurogenesis-mediated process(149), our results suggest that deficits in neuronal activity of mature and newborn neurons in the DG in the context of chronic intestinal inflammation, might result in abnormal function of the hippocampal circuitry which in the long-term disrupts the fine processes of memory storage.

4.4 Indication of innate immune memory induction in hippocampal microglia during acute and chronic DSS colitis

In the present study, we revealed increased infiltration of $CD45^{high/+}CD11b^{+}Ly6C^{+}$ myeloid cells, considered inflammatory, in the hippocampus during the 4^{th} and 7^{th} days of DSS-induced colitis, confirming interactions between peripheral inflamed tissues and the brain via cells of the innate immune system(351). The latter was supported by an increase in M1-like hippocampal microglia, a phenotype considered neurotoxic to adult NSCs, together with increased levels of pro-inflammatory TNF-α, IL-1β and IL-6, in the serum and hippocampus(240). Similarly, recent *in vivo* studies have shown that increased TNF-α signaling enhances the infiltration of myeloid cells in the brain(351). In contrast to the acute phase of colitis, switch of microglia to the M2 -like phenotype characterized the progress of the disease to its chronic phase(352). Considering the well-documented pro-survival and tissue-repairing effects of M2-like microglia, these findings raise the hypothesis that induction of M2-like microglia and increase in IL-10 levels observed during the progress of colitis could be associated with the increased survival and proliferation of NSCs in the hippocampus we identified in the chronic disease. In addition, astrocytes exhibited the immunosuppressive “M2-like” phenotype which is known to support neurogenesis. Astrocytes in the region of hippocampus have been shown to promote neuronal differentiation through the release of potent neurogenic factors(104). As chronic experimental colitis is an established disease with profound tissue injury but less mortality than the acute disease(83), one could argue that support of hippocampal neurogenesis by

microglia could be part of the adaptation response to the disease. In fact, microglia which reside in the hippocampus display a unique role in hippocampal neurogenesis, and, more specifically, in neuroblast survival(353). Recent studies have also shown that targeted ablation of DG microglia resulted in reduction in overall neurogenesis(353). Furthermore, whole genome analyses revealed that DG microglia exhibit a unique transcriptome profile, compared to other hippocampal regions (e.g. CA1)(353), in line with our findings showing increased numbers of $Iba1^{+}$ microglia in the DG during colitis-induced systemic inflammation.

In line with the aforementioned results, the transcriptional levels of the anti-inflammatory enzyme Arginase 1 were upregulated and accompanied by increased protein levels of the inhibited form of GSK3β kinase in the hippocampus. Apart from the known role of GSK3β in inflammatory responses(329), it has been shown that changes in the gut microbiome (e.g. treatment with antibiotics) increases the inhibitory serine-phosphorylation of GSK3 in the rodent brain, suggesting that alterations in the composition of the gut microbiota(354) which also occurs in IBD patients(355), influences the activity of GSK3 with possible effects on mood modulation and cognitive function(329). The inhibited form of GSK3β decreases NF-kB activation which is the key transcriptional indicator of pro-inflammatory gene expression(329). Indeed, this modulation was reflected in the protein levels of NF-kB in the hippocampus of mice with acute DSS colitis that was not altered further suggesting that acute DSS colitis induces an anti-inflammatory response in the adult hippocampus.
Activated microglia is known to induce several intracellular pathways which are implicated in the induction of inflammatory responses(330). For instance, the PI3K/AKT/mTOR pathway is known to be implicated in inflammatory responses, cellular activation and apoptosis(330). Acute DSS colitis induces upregulation of the protein levels of PI3K in the hippocampus with no increases in the activated phosphorylated form of mTOR. The activated form of mTOR increases the activity of NF-kB and promotes the expression of pro-inflammatory molecules including iNOS and COX-2(356). In our case, this was not evident in the hippocampus of mice with acute DSS colitis. These characteristics are indicative of resolution of acute inflammatory response and in line with the time course of the DSS-induced model of acute colitis.

The aforementioned findings are reminiscent of the recently-described processes of innate immune training and tolerance(301). It is now widely recognized that repeated exposure of innate immune cells to inflammatory stimuli can train cells to develop innate immune memory, in a process associated with epigenetic reprogramming. Notably, trained innate immunity does not only enhance protective immunity in response to subsequent exposure to pathogens, but can also affect stem cell biology(305). Indeed, a remarkable increase in the expansion of hematopoietic progenitors and myelopoiesis upon peripheral innate immune training was demonstrated in a very elegant study(305), whereas, in response to subsequent challenges such as, systemic inflammation and chemotherapy, innate immune training conveyed beneficial effects. Seminal studies have also documented that innate immune memory underlies microglia responses and shapes neuropathology in the long-term(304). More specifically, systemic administration of repeated doses of LPS induced microglia

training, followed by the establishment of immune tolerance that lasted up to six months(304). Trained microglia exacerbated the pathological outcome in response to secondary inflammatory stimuli, such as, during Alzheimer's- or stroke-induced pathology, while tolerant microglia could alleviate disease manifestations (304). In relevance to our results, we propose that innate immune training might occur early on during the acute phase of colitis, accompanied by enhanced neurogenesis, although with altered cell cycle kinetics of the proliferating progenitors.

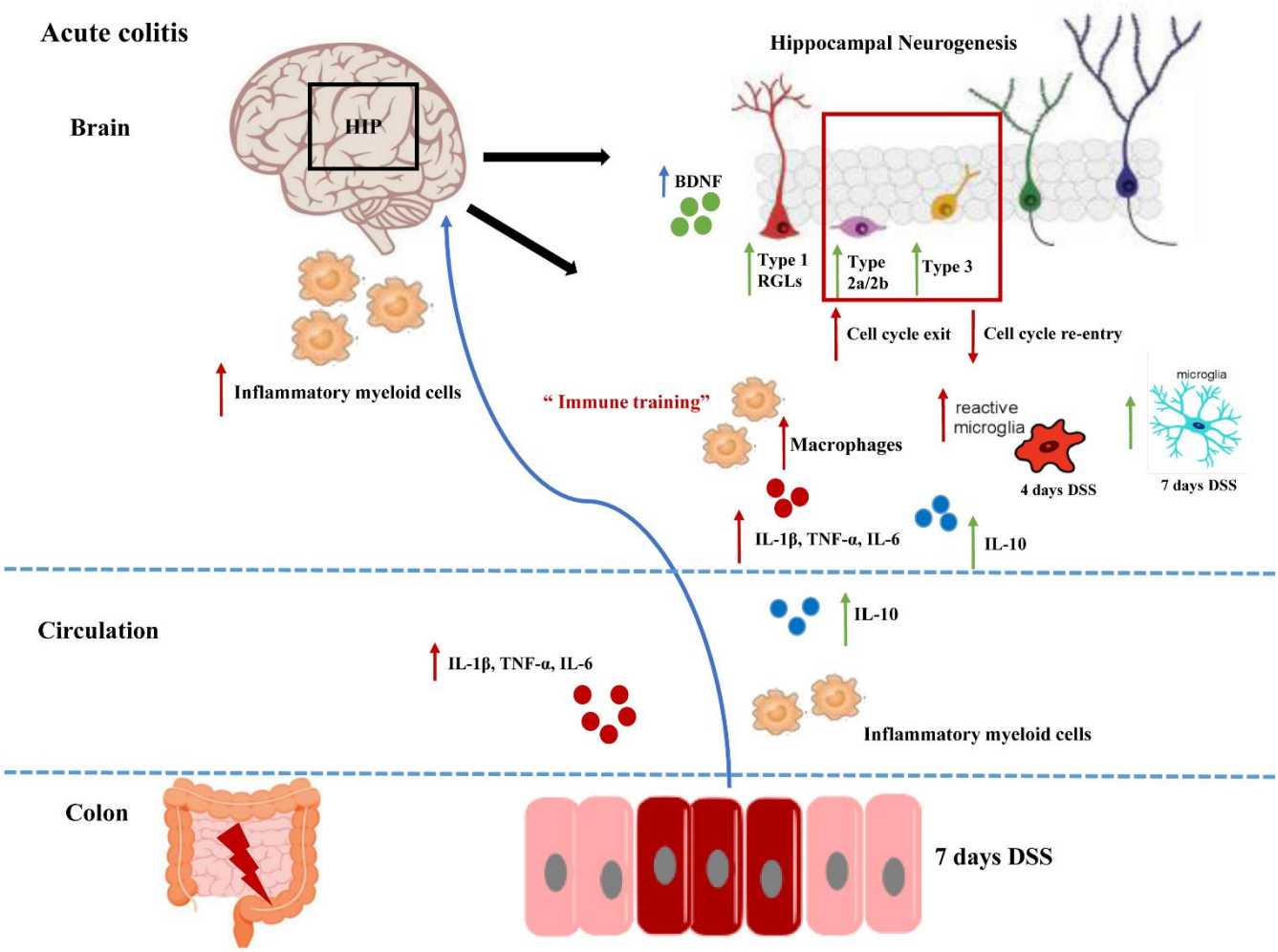

Figure 1. Indication of innate immune training of hippocampal microglia during acute DSS colitis. *Pro-inflammatory cytokines (IL-1β, TNF-a, IL-6) are increased in the serum of mice with acute DSS colitis with parallel increase of the anti-inflammatory cytokine IL-10 (immune training). Infiltration of inflammatory myeloid cells in the hippocampus was accompanied by activation of microglia and elevated levels of pro-inflammatory cytokines In addition, enhanced levels of the anti-inflammatory cytokine IL-10 in the hippocampus were accompanied by increased frequencies of the alternatively activated microglia (M2). The latter indicate the "training" response of microglia to peripheral inflammatory stimulus. These events were accompanied with enhanced hippocampal neurogenesis but with cell cycle deficits in Type 2a and Type 3 cells.*

In contrast, during the development of chronic colitis transition to an anti-inflammatory, immune tolerant, microglia phenotype occurs, which is accompanied by normal levels of hippocampal neurogenesis but deficient migration and integration of the newborn neurons. It is likely that the active neuroinflammatory environment early-on during acute DSS colitis might cause epigenetic alterations, as well as, changes in the metabolic processes supporting the proliferating progenitors, affecting the migration and functional integration of newborn neurons in the hippocampal circuitry. In support, changes in metabolic pathways, mainly in glucose metabolism and cholesterol biosynthesis, were observed in hematopoietic stem and progenitor cells in response to innate immune training stimuli(305). As metabolic alterations regulate the proliferation and differentiation processes of hippocampal progenitors(123), it will be of great interest to explore how intestinal inflammation coupled with training of microglia, affects the metabolism of neural progenitors in the DG.

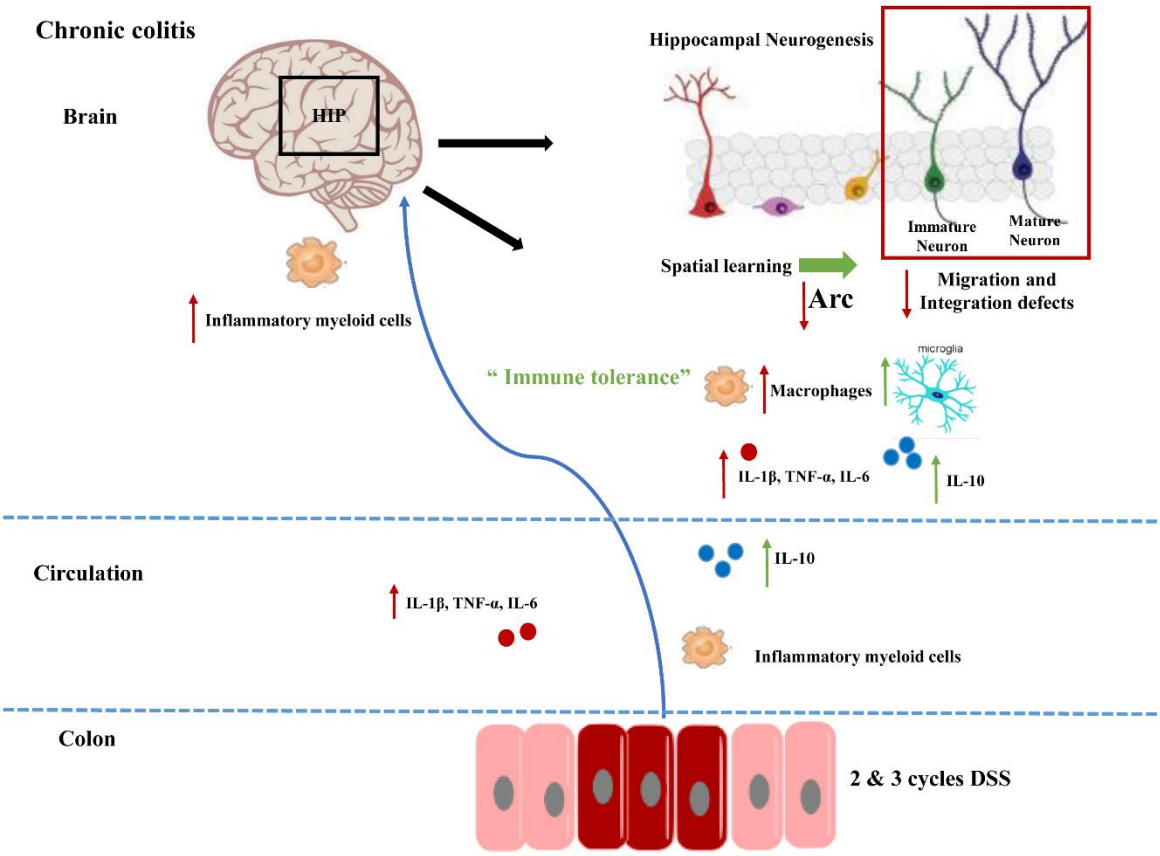

Figure 2. Indication of innate immune tolerance of hippocampal microglia during chronic DSS colitis. *Pro-inflammatory cytokines (IL-1β, TNF-a, IL-6) are decreased in the serum of mice with chronic DSS colitis with steady elevated levels of the anti-inflammatory cytokine IL-10 (immune tolerance). Infiltration of myeloid cells in the hippocampus is decreased while enhanced levels of M2-like microglia and IL-10 is evident in the hippocampus. These events were accompanied with migration and integration deficits. In response to a novel spatial environment, mice with chronic DSS colitis show decreased expression of the immediate early gene Arc both in mature and newborn neurons of the DG*

4.5 Proposed mechanisms that mediate the effects of experimental gut inflammation on hippocampal neurogenesis and microglia

A bottom up approach should be addressed to study the molecular mechanisms involved in the regulation of hippocampal neural stem cells and glia physiology during intestinal inflammation and the subsequent behavioural outcome. The activated immune system and alterations in the gut microbiome are potential targets for understanding the mechanisms of the bidirectional communication between the gut and the brain as well as for developing novel pharmacological interventions. Recent studies have shown that immune system functions in tight conjunction with the gut microbiome and regulate the response of the innate immune system of the brain. For instance, a recent study reported that plasma cells (white blood cells that originate as B cells in the bone marrow) that reside in the gut and produce Immunoglobulin A (IgA) antibodies migrate to the CNS and produce an anti-inflammatory effect during the active phase of multiple sclerosis(357). Depletion of $CD4^+$ T lymphocytes induces reduction of hippocampal neurogenesis and impairments in hippocampus related behavioural tasks(357). Reconstitution with $CD4^+$ cells that are non-CNS specific and rarely detectable in the healthy brain in mice which lack T cells restore neurogenesis(248). These data suggest that CD4-T cell activity is required for hippocampal neurogenesis and neuronal plasticity. Perturbations in the gut microbiota due to antibiotic treatment exert debilitating effects on hippocampal neurogenesis which is rescued with voluntary exercise, probiotic treatment and adoptive transfer of monocytes ($Ly6C^{hi}$ monocytes)(293). Specifically, this monocyte population is crucial for brain homeostasis and plasticity(293). Moreover, gut microbiome is a major regulator of microglia function and morphology through gut derived molecules such as short fatty acids (SCFAs)(294). An altered microglia function can lead to neuroinflammation and negative regulation of hippocampal neurogenesis with debilitating behavioural outcome in memory and learning processes.

These studies give us a hint of possible mechanisms that explain the observed effects on hippocampal glia and neurogenesis in DSS induced colitis. The anti-inflammatory response in the hippocampus of mice with acute and chronic DSS colitis could probably induced by plasma cells or other type of T cells that migrate in the CNS from the inflamed gut. This can partially explain the non-response of microglia and the enhanced neurogenesis. Using T-cell deficient mice and adoptive transfer of various gut derived T and B cells during acute DSS colitis we can explore their contribution in the regulation of microglia and neural stem cells of the hippocampus. This experimental strategy cannot be performed in the chronic model of DSS colitis as the adaptive immune system is essential to counteract the persistent inflammation. In this case, Cre-Lox system that target a specific subpopulation of T and B cells can be used to study their contribution in glia and neural stem cells physiology in specific time points of chronic colitis.

Dysbiosis refers to an altered gut microbiome and is evident in murine models of colitis(355). It has been shown that alterations in the gut microbiome affects microglia function(294), neuroplasticity mechanisms(358) and neural stem cell biology(296). Alterations in the gut microbiome affect BBB permeability(19), tryptophan metabolism(9) and intestinal immune response. Persistent intestinal inflammation in conjunction with an

altered microbiome profile can affect the integrity of BBB thus facilitating the entry of bacterial substances (e.g LPS), inflammatory cues and immune cells. This can potentially activate the local microglia and cause neuroinflammation. In our model BBB permeability could be affected in the chronic model of colitis where microglia is activated and deficits in the migration and integration of newborn neurons are evident. Most interestingly, due to alterations in the microbiome profile and intestinal inflammation, tryptophan metabolism can be affected via the activation of the enzyme IDO and signaling via the kynurenine pathway(359). IDO is activated in IBD and in murine models of colitis(359) and it is also implicated in major depression(360). In the brain, IDO is primarily expressed by microglia and astrocytes(361). Activation of IDO by cytokines and bacterial substances such as LPS diverts the metabolism of the tryptophan in the brain from the pathway culminating in the neurotransmitter serotonin to an alternative metabolic pathwa(362)y termed kynurenine pathway (KP)(362). The products of this pathway have divergent effects and stimulate NMDA receptor signaling(362). These effects can lead to depressive like behaviors in mice and blockade of KP activation ameliorates these detrimental effects. In DSS experimental colitis, pharmacological inhibition of IDO by L-1MT (1-methyl-D,L-tryptophan) attenuated the severity of DSS-colitis(363). Also, the disease in $Ido1^{-/-}$ mice was less severe than in $Ido1^{+/+}$ mice(363). To our knowledge there are no data available regarding the activation status of IDO in glial cells of the brain during acute or chronic DSS colitis. This can unravel a novel mechanism that link the gut and the brain during intestinal inflammation and might refer as a potential target for pharmaceutical treatment.

4.6 Future Perspectives

As the mechanisms that mediate the effects of intestinal inflammation on the brain are largely unknown, it is of great interest to unravel the mechanistic roots implicated in the response of microglia and neural stem cells in the hippocampus under acute or chronic intestinal inflammation. To do so, we propose a series of future experiments dissecting the role of various roots of gut brain axis communication in response to intestinal inflammation.

The vagus nerve is one route of neuronal communication between the intestine and the brain(8). To investigate whether the vagus nerve is involved in microglia and neural stem cell activation in the hippocampus under acute or chronic intestinal inflammation, vagotomy can be performed either before or after acute and chronic administration of DSS. As IBD exert a robust systemic pro-inflammatory profile(323), treatment with common anti-inflammatory drugs (e.g. minocycline) can reveal if cytokines are the major mediators of the observed effects on microglia and neural stem cells activation. Notably, we can specify if these effects are TNF-a, IL-6 or IL-1β-mediated, by performing treatments with humanized antibodies during acute or chronic DSS colitis. Such interventions might have translational impact, as these treatments are commonly used in the treatment of IBD (364). Also, these treatments can be used to monitor changes in behaviour, especially in mood, learning and memory tasks.

We previously discussed that patients with IBD present with dysbiosis(355) and absence of specific microbiome metabolites (e.g. SCFAs). To see if the microbiome plays a role in the activation of neural stem cells and microglia during DSS colitis, studies with Germ free or

SPF (specific pathogen free) mice(365), might evaluate the microbiome contribution to the observed effects. Furthermore, reconstitution with flora derived from wild type recipient mice or delivering specific microbiome metabolites (e.g. butyrate) can further validate the link with the gut microbiome, hippocampal neurogenesis and microglia activation.

Microglia are key regulators of adult hippocampal neurogenesis(256). Ablation of microglia either pharmacologically by treatment with PLX3397 (inhibitor of the survival receptor CSF1)(366) or genetically by using $Cx3cr1^{CreERT2}$;$DT^{loxP/loxP}$ mice can reveal if microglia are the main mediators on the observed effects on adult hippocampal neurogenesis during acute or chronic DSS colitis. Moreover, the use of CX3CR1-CreER (Cre) mice crossed with mouse lines carrying loxp-flanked genes (e.g Tak1 or Hdac1/2) can be used to study the potential acquired innate immune memory of microglia in the hippocampus during acute and chronic DSS colitis. This can be accompanied with studies on the metabolism of microglia and adult neural stem cells under acute or chronic intestinal inflammation.

4.5 Conclusions

In summary, our study provides mechanistic insights into colitis-induced inflammatory processes in the brain that impinge on hippocampal neurogenesis. These findings may have important implications for IBD patients, as they suggest that early detection of DG dysfunction might be a valuable indicator of the development of associated cognitive deficits and depression. Adult hippocampal neurogenesis can be targeted as treatment for depression and anxiety in IBD patients. For that, IBD patients with neurogenesis-dependent impairments in DG function will first need to be identified. This can be achieved with imaging techniques of cerebral blood volume (CBV) in the DG, which has been shown to correlate with levels of neurogenesis, positron emission tomography (PET) using neurogenesis markers and magnetic resonance spectrometry (MRS)(367). Also, neurocognitive testing could be used to identify IBD patients with DG-dependent cognitive impairments such as pattern separation tasks or virtual reality memory tasks with a spatial navigation component(368). Determining impairments in these tasks can potentially lead to treatments that increase neurogenesis such as exercise, cognitive and learning strategies as well as the use of selective serotonin reuptake inhibitors (SSRIs)(369). Enhancing neurogenesis and improving DG function in IBD patients with confirmed impairments may increase pattern separation, cognitive flexibility and reduce anxiety levels through regulation of the HPA axis. Treating these impairments may facilitate remission in IBD patients and counteract the development of depressive and anxiety behaviors observed in these patients. Therefore, the development of targeted treatments that can alter neurogenesis, mood and cognition disturbances, such as, anti-depressants, physical exercise and other interventions with suggested beneficial effects for neurons, should be considered as valuable tools for the overall management of IBD patients.

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