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Microglia-complement implication and structural-functional synaptic characterization in the hippocampus of early Experimental Autoimmune Encephalomyelitis (EAE) model of Multiple Sclerosis

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by

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Abstract

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS) in which the immune system plays a central role to drive pathology. In addition to motor and sensory symptoms, memory impairment is reported in MS patients in early stage of the disease and in rodent models prior to the disease onset.

The synaptic pruning is a physiological developmental mechanism, crucial for brain connectivity in which the complement components C1q-C3 and the phagocytic receptors expressed by microglia, C3R and Trem2, have been implicated. A reactivation of similar mechanism may be one of the central causes of the synapses loss in neurodegenerative disorders including MS and might explain the early synaptic changes occurring within the hippocampus.

In this thesis, we have analyzed the demyelination process, the classical complement components activation and the synapse-microglia interaction in the hippocampus of experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We have focused on the earliest stage (i.e. after induction), when motor symptoms are still not observed. Furthermore, we have investigated the role of microglia phagocytic receptors Trem2 and C3R and the classical complement component C3 in the disease progression.

Following immunohistochemical analysis, we have found no demyelination or microglial activation, as well as unaltered complement components expression levels. In addition, we have found no changes in the synaptic density within the hippocampus of the asymptomatic EAE

mice, compared to their controls. Notably, we have found an increased VGAT expression in the CA1 region of the hippocampus at day 8 post-immunization which reflects a subtle alteration of inhibitory neurotransmission but whose significance is still unclear.

Moreover, electrophysiological examination of the hippocampal synaptic transmission and plasticity did not establish any changes on synaptic function at the earliest stage after induction. However, genetic deletion of complement protein C3 and its receptor subunit CD11b reduces motor symptom severity in EAE mouse but has no effect on the disease onset. We then sought to determine the role of Trem2 receptor on clinical motor score severity. Unexpectedly, inducing EAE in Trem2 knockout mice is associated with a reduction in clinical score and in the demyelination progression.

To sum up, we believe that hippocampus synaptic degeneration occurs late in the disease, when motor symptoms are well established. Overall, we have confirmed the role of classical complement components C3 and C3R in the EAE progression and disease manifestation, and we have identified Trem2 receptor as a potential anti-inflammatory target for a disease modifying therapy.

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List of abbreviations

ACSF = artificial cerebrospinal fluid

AD = Alzheimer disease

AMPA = α-amino-3- hydroxy-5-methyl-4-isoxazole-propionic acid

APCs = antigen-presenting cells

BBB = the blood brain barrier

BDNF = Brain-derived neurotrophic factor

BS = basal synaptic transmission

C3R = complement C3 receptor

CFA = Freund's complete adjuvant

CNS = central nervous system

CO = carbon monoxide

CPZ = cuprizone

CR = complement receptors

CSF = cerebrospinal fluid

d.p.i= day post-immunization

DAMPs = danger-associated molecular patterns

DAP12 = DNAX adaptor protein-12

DG = Dentate gyrus

EAE = Experimental autoimmune encephalomyelitis

EBV = Epstein-Barr virus

EC = entorhinal cortex

E-LTP = early phase of LTP

ERK= extracellular signal-regulated kinase

fEPSP = excitatory postsynaptic potential field

HFS = high- frequency stimulation

HLA = The human leukocyte antigen

i.p = intraperitoneally injection

I/0 = Input/Output

Iba-1 = ionized calcium-binding adaptor molecule 1

IFN-γ = interferon gamma

iGluRs = glutammate receptors

IHC = immunohistochemistry

IL = interleukin

KO = knock-out

L-LTP= Late phase of LTP

LPS = lipopolysaccharide

LTD = long-term depression

LTP = Long term potentiation

MBL = mannose-binding lectin

MBP = Myelin basic protein

MHC = the complex major histocompatibility

MOG = the myelin oligodendrocyte glycoprotein

MOG 35–55 = myelin oligodendrocyte glycoprotein, peptide 35–55

MPP = medial perforant path

MS = multiple sclerosis

NDS = normal donkey serum

NMDA = N-Methyl-D-aspartate

NO = Nitric oxide

NOS2 = nitric oxide synthase-2

PAMPs = pathogen-associated molecular patterns

PD = Parkinson disease

Poly I-C = polyinosinic polycytidylic acid

PP = perforant pathway

PPD = paired-pulse depression

PPF = paired pulse facilitation

PPMS = primary progressive

PPR = paired Pulse ration

PRMS = progressive relapsing

PRRs = pattern recognition receptors

PTX = Pertussis Toxin

ROS = reactive oxygen species

RRMS = relapsing remitting

SC = Schaffer collateral pathway

SPMS = secondary progressive

STD = short term depression

STP = short term plasticity

sTrem2 = soluble variant of Trem2

TBS = theta burst stimulation

TH =T helper

TLRs = Toll-like receptors

TNF α = tumor necrosis factor alpha

TREM2 = triggering Receptor Expressed On Myeloid Cells 2

WT = wild type

1. Introduction

1.1. Multiple sclerosis

Multiple Sclerosis (MS) is an autoimmune inflammatory disease of the CNS, believed to be caused by genetic and environmental factors interaction (Compston and Coles, 2008). Demyelination, inflammation, neuronal loss and axonal damage represent the major hallmarks of the disease (Popescu et al., 2013) which lead to a wide range of symptoms that negatively impact the quality of life of MS patients including the social and economical sides.

MS can be described by two main clinical phenotypes: relapsing and progressive disease, which include 4 subtypes; relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS) and progressive relapsing (PRMS) MS according to the experienced symptoms and the severity of disease progression (Lublin et al., 2014). The first observed neurological symptoms and single or multiple lesions in the brain or spinal cord are defined as a clinically isolated syndrome (CIS). If the attacks are repeated with an intermittent periods of exacerbations (relapses) and recovery (remission) then the diagnosis of the disease is known as the relapsing remitting MS (RRMS), the most common worldwide form of MS. The symptoms may worsen over the time with less or without relapse and remission then the RRMS evolve to secondary progressive MS (SPMS). Another form of MS is the Primary-Progressive MS (PPMS), characterized by a slow progression in disability from the beginning without episodes of relapses and remissions, occurring in about only 15% of patients in which it may evolve into the Progressive-Relapsing form (PRMS) , a rare form characterized by a consistent

progression in the neurological and physical symptoms with acute relapses without remission (Cavoni e al., 2012;Lublin et al., 2014) (Figure 1.1).

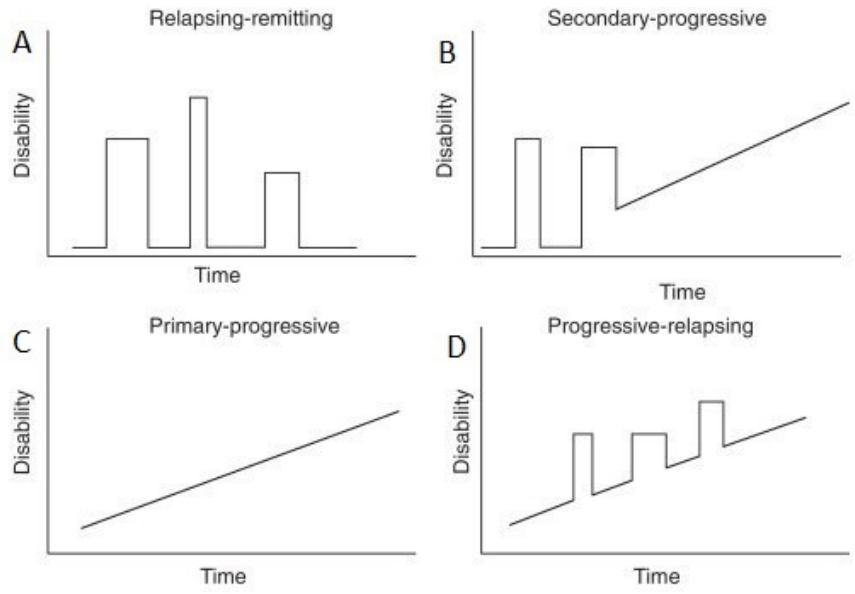


Figure 1.1 clinical courses of multiple sclerosis

A. Relapsing-remitting MS: lack of disease progression; periods of relapses are followed by remission in which symptoms disappear . B. Secondary progressive MS: disability gets worse over time, characterized by progression with or without relapses and less recovery. C. Primary progressive MS: Disease progression from the start, with no episodes of relapses and remissions. D. Progressive-relapsing MS: steadily progression of the disease with relapses without improvements (Cavoni et al., 2012).

1.1.1. Clinical features and risk factors

Neurological deficits in MS manifests in various clinical manifestations, depending on the location of demyelinating lesions in the gray and white matter of the CNS. For example, lesions in the corticospinal tract in the spinal cord are associated with motor symptoms such as weakness,

paresis and paraplegia, whereas sensory symptoms in MS including numbness, tingling, burning are related to those of the myelinated axons in the spinothalamic tract or of the posterior columns (Rahmanzadeh et al., 2018). In addition, 40-65% of patients with MS develop impairments in cognitive functions such as deficits in attention, memory processing and spatial abilities which are associated mainly with cortical lesions and atrophy of the gray matter (Jongen et al., 2012).

The exact cause of MS is unknown. However, The risk of developing the disease depends on two prominent risk factors and their interaction (Ramagopalan et al., 2010).

Environmental: three principal factors were identified as a possible causes for the development of MS in the early adulthood i.e. Epstein-Barr virus (EBV) infection, lack of vitamin D, and smoking behaviour.

In a dose/exposure dependent manner, Cigarette smoking has a different negative effects toward the immune system which were associated with the incidence of MS in vulnerable patients (Hedstrom et al., 2009). The Tobacco contains high amounts of toxic compounds and induces the release of multiple proinflammatory cytokines, for example, high levels of nitric oxide (NO) and carbon monoxide (CO) affect tissue oxygenation and may induce a persistent immune system activation leading to neurotoxicity (neurons death, oligodendrocyte necrosis, axonal degeneration and demyelination) and tissue damage (Alrouji et al., 2019). Additionally, Smoking affects the course of the disease in MS patients. Several studies suggest that the probability of worsening on symptoms, the increase in demyelinating plaques and the transition to a more

aggressive form of MS i.e. the SPMS are increased a year by year in tobacco users rather than non-smokers (Hernán et al., 2005).

Epstein-Barr virus (EBV) infection among teenagers and adults is one of the strongest risk factor which is believed to trigger MS pathogenesis in genetically susceptible individuals, due to the correlations between the disease and seropositivity for the EBV in MS patients and the studies on viral-induced animal models of neuroinflammation, demyelination, and neurodegeneration (Levin et al., 2003; Lassmann and Bradl, 2017). EBV, also known as human herpesvirus 4 (HHV-4), is a gamma-herpes virus. Entering the body, EBV is enable it to evade the immune system and induces an acute inflammation by infection B cells. Establishing a lifelong latent infection with occasional or no reactivation inside the body (Ressing et al., 2015).

Among the hypotheses that have been proposed to explain the increased risk of developing MS in relation to EBV, the one that explain how EBV is implicated in the triggering of both peripheral and CNS immune system contributing to the activation and immigration of the immune cells within the CNS resulting in the progression of MS disease (Bar-Or et al., 2020). Another theory suggests that the involvement of EBV in MS pathogenesis is possibly due to the enhanced permeability of the blood brain barrier derived by the acute primary infection, contributing to the onset of the disease (Casiraghi et al., 2011).

The latitude distribution of MS and its prevalence in area distance from the equator characterized by low UVB exposure, has suggested the importance of vitamin D insufficiency as a possible environmental risk factor (Beretich et al., 2009). Indeed, a number of researches have shown

that the exposure to vitamin D through exposure to ultraviolet B radiation (UVB) or through a diet (milk, salmon, Vitamin D2 supplements…), is associated with lower risk of MS (Orton et al., 2011).

Vitamin D has a potential effect in maintaining immune response homeostasis, by exerting a physiological protective role in limiting potentially pathological over-activated immune responses mediated the control of macrophages, dendritic cells and Lymphocyte T and B proliferation, differentiation, and activation, providing a plausible evidence for its in autoimmune disorders including MS (Mora et al., 2008). In effect, given to its immunomodulatory properties, vitamin D obtained from exogenous sources as a putative immunotherapy offered benefits effect on mouse model of MS by inhibiting the disease onset and progression (Cantorna et al., 1996; Pederson et l. 2007), and in to people who already have been diagnosis with MS (Sotirchos et al., 2016).

Genetic predisposition: In Concordance studies in monozygotic twins, genetics factors are suggested to be involved in the risk of developing MS (Willer et al., 2003). The one major dominants risk factors are genes within the human leukocyte antigen (HLA) complex. HLA are a cluster of genes located on chromosome 6p21, which encode for the major histocompatibility complex (MHC) class I, II and III which play a pivotal role in driving the immune response mediating the antigen presentation to the adaptive immune cells (Wieczorek et al., 2017). Many of allele variants corresponding to class II were founded to be a strong risk allele associated with MS development such as DRB1*15:01, DRB5*01:01, HLA-DRB1*15:01 and HLA-DRB1*13:03 (Patsopoulos et al., 2013; Moutsianas et al. 2015). Whereas, some haplotypes such as HLA-A*02:01, HLA-B*44:02

corresponding to class I variants are considered to be protective against MS (Moutsianas et al. 2015).

1.1.2. Microglia involvement in the pathophysiology of the disease

Microglia, the resident macrophage-like cells of the CNS, represent the main effector cell of the innate immune response following the CNS infection, lesions, trauma and neurodegenerative processes. As one of the first defense barrier, microglia produce and release pro- and/or anti-inflammatory cytokines and several neurotrophic factors and are involved in the removal of cells debris in order to restore the CNS homeostasis (Neumann et al., 2009; Li and Barred, 2018).

Microglia cells originate from the yolk sac and migrate in the CNS during embryonic development to form the resident immune cells (Davous et al., 2008). Throughout life, they maintain their intrinsic ability to repopulate themselves in order to keep maintain the normal physiology of the CNS (Ajami et al., 2007). Microglial cells are characterized by have a very large potential activity to maintain the CNS homeostasis. First of all, they play a key role in the proper development of the CNS due to their phagocytic activity which allows the elimination of apoptotic neurons during the embryogenesis, an important process to build and maintain the neuronal connections (Paolicelli et al., 2011). In addition, As immune cells, they act as sentinels, detecting the first signs of pathogenic invasion or tissue damage providing the first line of defense of the CNS. ln the healthy brain, microglia are found in a resting state distinguished by its particular morphology characterized by a small cell body with fine ramified processes which extend in multiple directions, and low expression of

immune related cell surface receptors (Kierdorf and Prinz, 2017). Perturbation within the CNS can induce a rapid transition of the microglia state from resting/quiescent to activated influenced by the nature of the insult. Following their activation, they undertake changes in both their morphology adopting an amoeboid profile with shortened processes that facilitate both their division and their movement to the site of the injury, and in their phenotype by the modulation of the expression of the membrane, cytosolic and extracellular immune related proteins. In the injury site, microglia are recruited either directly by the pathogen itself or by chemokines released by other affected neuronal cells (Napoli and Neumann, 2009; Gómez-Nicola et al., 2013). Thus, a nomenclature were adopted for the states between which the microglial cells oscillate: a classic activation state (M1) and an alternative activation state (M2) (Figure 1.2) (Mantovani et al., 2013).

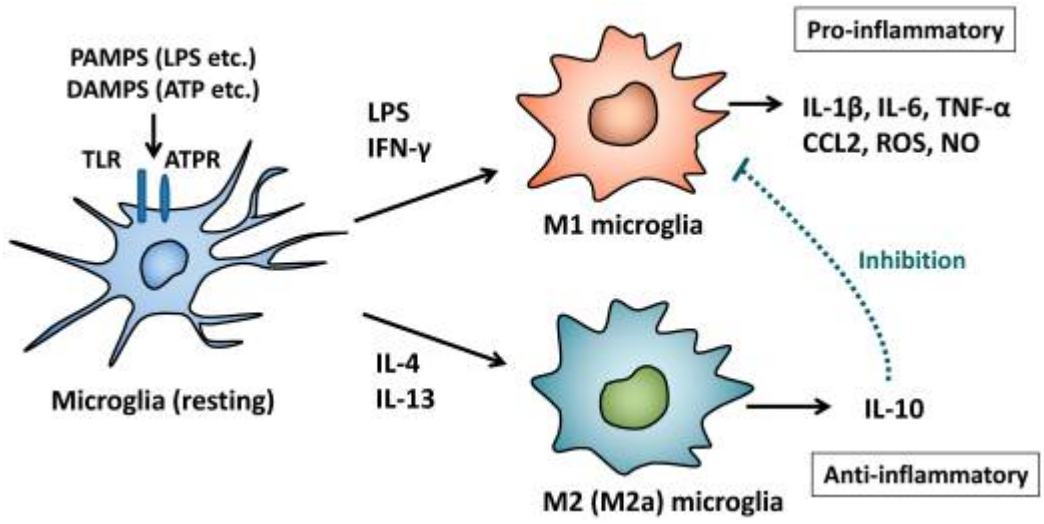


Figure 1.2 The classical and alternative activation of microglia and their immunoregulatory functions

Resting microglial cells are stimulated with PAMPS or DAMPS via TLR or ATP receptors. The polarization of the microglia to the classical activation (M1), considered neurotoxic, in response to the LPS and INF -γ is characterized by the release of several pro-inflammatory and neurotoxic molecules such as ROS, NO, TNFalpha, IL-6, IL-1beta, CCL2. On the contrary, the neuroprotective alternative activation (M2) is triggered upon exposure to anti-inflammatory cytokines IL-4 and IL-13, and It is characterized by an increased expression of anti-inflammatory molecule such as IL-10 (Nakagawa and Chiba, 2014).

To monitor the environmental changes in the CNS, under pathological conditions, microglia express numerous receptors for the various families of neuronal signalling molecules which monitor their "on"/ "off" state and respond to these stimuli with high specificity (Biber et al., 2007). These transitions from a state of surveillance to that of activation are triggered when the microglia perceives the sudden appearance of pathological factors such increased neurotransmitters release, entry of microorganisms, etc.

Microglial cells express receptors for neurotransmitters such as glutamate, GABA and dopamine which leads to the hypothesis that microglial cells detect neuronal activity via the local level of neurotransmitter in their environment. In addition, changes in neurotransmitter concentrations would influence microglial functions in order to induce a response adapted to the context (Liu et al., 2016). For example, Glutamate, the main excitatory neurotransmitter, if released in excess under pathological conditions exerts excitotoxic effects which is an "on" signal for microglial activation, The activation of the glutamatergic ionotropic receptors AMPA, Kainate and NMDA receptors expressed by microglia modulates different cellular functions: motility, factor release, induction of cell death, ... (Liu et al., 2009). However, activation of certain microglial glutamatergic

receptors has neuroprotective effects (Liu et al., 2016). Additionally, the activation of microglial cells can be regulated by the cytokines present in the CNS. Cytokines are a large category of signaling molecules involved in cell signaling and regulation of the inflammation process, synthesized by cells of the immune system or by other cells or tissues (Zhan and An, 2007). Six major classes of cytokines are found in the CNS, i.e. ILs, TNFs, IFNs, chemokines, then CSF (colony stimulating factors) (Szelényi, 2001). Microglia influence neuronal survival by releasing a variety of ILs that modulate the functions of surrounding toxic or beneficial immune cells. Among which, IL-I α, IL-I β, and IL-6 a pro-inflammatory propriety, while IL-4, IL-I0 and IL-13 are immunosuppressive (Czeh et al, 2011). For example, IL1 β, a pro-inflammatory" cytokine, is constitutively expressed by different cells including the microglia at a low level under physiological conditions. Indeed, IL-1β has important functions including the regulation of the sleep (Imeri and Opp, 2009) and the modulation of the synaptic plasticity (Bellinger et al., 1993; Ross et al., 2003; Moore et al., 2009). However, under pathological conditions, sustained overexpression of IL1 β by microglia cells increase inducing excitotoxic neuronal damage and impairs contextual and spatial hippocampus dependent memory (Centonze et al., 2009; Nistico' et al., 2013). An additional key cytokine involved in the microglia activation in the healthy and perturbed brain is the IFN γ. Indeed, the stimulation of cells by IFN γ triggers the expression of the proteins of the complex major histocompatibility (MHC) of classes I and II setting their functions of detection and presentation of antigens to T cells (Xu and Ling, 1994). Moreover, in vitro exposure of microglia cells to IFN γ increases their capacity for phagocytosis, secretion of enzyme proteolytic and the production of Reactive oxygen species (ROS) (Dheen et al., 2007; Tambuyzer et al., 2012). An additional important class of

receptors involved in microglia activation are Toll-like receptors (TLRs), members of the family of “pattern recognition receptors” (PRRs) evolutionally conserved which recognize exogenous pathogenic molecular structures (the PAMPs for "pathogen-associated molecular patterns"), but also endogenous ligands released under pathological conditions (DAMPs for "danger-associated molecular patterns ”), connecting the innate immune responses via detection of pathogens and induction of inflammatory response and the adaptive immune response via recruitment and activation of T cells (Kaisho and Akira, 2006). Microglia can express all TLR family members. Further, it was reported that activation of microglia by, in particular by the polyinosinic polycytidylic acid (Poly I-C), lipopolysaccharide (LPS) which are TLR3 and TLR4 agonists respectively, induces the secretion of pro-inflammatory cytokines, such as TNF- α (tumor necrosis factor alpha), and the increase in phagocytosis, chemotaxis, oxidative stress and induction of nitric oxide synthase (OIson and Miller, 2004).

Microglial cells can be activated by the stimulation of complement receptors (CR). Microglial cells express the CR 1, 3 and 4 in activity-dependent manner (Crehan et al., 2012). This system is made up of a number of circulating and associated proteins membranes that act synergistically to activate a proteases cascade which, mainly, promotes the phagocytosis of pathogens or cell debris to protect against autoimmunity. In addition, it has been shown that the CR3 (CD11b, CD18), often used to label microglial cells in the mouse CNS, would allow direct recognition of certain bacterial patterns, in particular LPS and therefore induce rapid activation of cells expressing it including the microglia (Ehlers, 2000). In neurodegenerative diseases, it has been reported a strong expression of

complement components and overactivation of microglia cells, associated with a high rate of neuronal death preceded by aberrant synaptic function and synaptic loss. As a pathological mechanism, it has been proposed that the complement proteins marks the affected synapses for elimination through the activated microglia (Perry and O'Connor, 2010). This mechanism will be further explained in the next section (1.1.3).

Trem2 (triggering receptor expressed by myeloid cells-2), a membrane-bound molecule and its associated signaling molecule DAP12 (DNAX adaptor protein-12), are involved in the control of microglial activation in the healthy brain (Schmid et al., 2002). The Trem2 / DAP12 complex plays an important role in the regulation of the inflammatory response and in the elimination of apoptotic cells. The microglial Trem2 has been shown to recognize, although not yet well identified, a Trem2 ligands (Trem2-L) expressed by apoptotic cells and promoting phagocytosis (Hsieh et al., 2009). In addition, inhibition of microglial expression of Trem2 reduces phagocytosis apoptotic neurons and increases the expression of TNF α and NOS2 (nitric oxide synthase-2) (Takahashi et al., 2005).

In summary, microglia cells are activated and monitored by a variety of proteins and receptors, and are able to activate and/or inhibit a wide variety of signaling molecules which allow them to quickly sense and detect the perturbation that could transform the CNS from a physiological to a pathological state. However, their activity may be beneficial or harmful depending on the persistence of the stimulus and their state of activation.

Under physiological conditions, microglia play a critical role in maintaining CNS homeostasis by exerting different functions.

Dynamic and constant monitoring of the brain environment: The use of transgenic mice expressing GFP under a microglia promoter allowed to study the behavior of these cells in vivo. In fact, the highly long branched microglia processes are very mobile and in a constant cycles of rapid retraction / extension suggesting permanent reorganizations of the cytoskeleton to converge rapidly on the site of a possible injury without cell body movement, keeping under control the health of the tissue (Davalos et al., 2005). This dynamic behavior of microglial extensions is dependent on signaling mediated by P2Y12 microglial receptors activation by the ATP and by the signaling pathway, fractalkine / CX3CR1, in which are involved the neurons responses (Ohsawa et al., 2010; Lauro et al., 2019)

Regulation of the adult hippocampal neurogenesis: In the adult brain, neurogenic niches have been identified in the subventricular zone located under the ventricles lateral and in the dentate gyrus (DG) of the hippocampus which are able to produce new neurons throughout life (Taupin, 2006). During neurogenesis, only a small number of newly created cells survive and are incorporated into the neuronal network and the remain immature cells go through the apoptotic process. This fine distinction and balance between the differentiated and the apoptotic cells and in the adult hippocampus is regulated by the microglial inflammatory and phagocytic activity (Sierra et al., 2010). Alternatively, microglia activated by anti-inflammatory cytokines IL-4 and INF γ were shown to promote neurogenesis (Butovsky, 2005).

Modulation of the long term synaptic potentiation (LTP): Growing evidences support the key role of the microglia on synaptic plasticity in the mature CNS via the regulation of LTP process. LTP is considered to be the cellular and molecular model that underlies learning and memory

(Richter-Levin and Yaniv, 2001). In the healthy brain, the neuron / microglia signaling pathway (fractalkine / CX3CR1) is involved in synaptic transmission and plasticity, mice knock-out (KO) in the CX3CR1 showed defects in hippocampal LTP induction and impaired associative and spatial memory, due to a overproduction of the pro-inflammatory cytokine IL1 β by activated microglial cells. (Rogers et al., 2011). Moreover, a disruption of another neuron / microglia signaling pathway, the CD200 / CD200-R pathway, is associated with impaired LTP (Costello et al., 2011). Furthermore, The cytokine, TNF-α, released by glial cells, enhances synaptic efficacy by increasing surface expression of AMPA receptors in neuronal cultures (Beattie et al., 2002)

An environment with less activates microglia, or overactivated, might give rise to a reduction of trophic factors production or excessive inflammatory molecules production, which have long been reported in different neurodegenerative disease including the Alzheimer disease (AD), the Parkinson disease (PD) and MS (Stephenson et al., 2018).

Studies on post-mortem MS brains reported how the microglia in active and chronic MS lesions produce a variety of pro- and anti-inflammatory molecules, which have been attributed both detrimental and neuroprotective functions (Cao and He, 2013; Voet et al., 2019).

Microglia cells in the early active lesion stages are expressed in M1 state, participating to the initial acute inflammatory response and in the recruitment of Lymphocyte T (Magnus et al. 2005). However, in the later stage, the M2 phenotype, which is the phenotype that is associated with resolving inflammation, cleaning of the myelin debris, promoting of remyelination process and axonal regeneration, become predominant (Mikita et al. 2011; Voss et al. 2012). This M1/M2 phenotype balance plays

an important role in disease progression and it is believed to be altered in MS which explain the continues progress of the disease and the tissue damage (Mikita et al. 2011).

Protective role of microglia in MS: To restore CNS homeostasis, microglia produces variety of beneficial factors including the IFNβ, which, is an approved treatment for RRMS (Limmroth et al., 2011). IFNβ secreted by microglia nearby to the active demyelinating lesions induces an increasing in itself phagocytic activity mediating a high efficiency in the myelin debris clearance (Kocur et al., 2015). Remyelination is the process of myelin regeneration, which is characterized by the formation of new myelin around the demyelinated axon, the phagocytosis of myelin debris and activation, proliferation and migration of oligodendrocytes are the key processes (Franklin and Ffrench-Constant, 2008), aberrant function of microglia/macrophages by removing CX3CR1 ,in demyelinating animal model, reduces myelin debris clearance and impairs remyelination process (Lampron et al., 2015). Moreover, it was found that microglia produced TNF-α and The growth factors IGF-1/ FGF-2, which have a crucial role for the proliferation of oligodendrocytes (Voss et al., 2012).

In EAE mice, microglia upregulate the production of the anti-inflammatory cytokine IL4, essential to maintain the balance between pro- and anti-inflammatory signal. Moreover, EAE mice treated with il-4 reported less severe disease and demyelination (Ponomarev et al., 2007; Racke et al., 1994; Zhang et al., 2014). Moreover, several studies have shown the beneficial effect in microglial depletion, especially in EAE models of MS, by the use drugs such as tetracycline or dipyridamole. The treated animals showed a delayed onset of EAE and less severe symptoms (Popovic et al. 2002; Sloka et al. 2013).

Detrimental role of microglia in MS: One of the hallmarks of active MS demyelination lesions is the accumulation of the phagocytic cell including the microglial containing even before the myelin degradation. Therefore, a harmful role of microglial cells and their contribution in the initiation of MS have been already proposed. Microglial activation in MS has been associated to direct impairment of neuronal and synaptic integrity through different mechanisms. Increasing in the mitochondrial NADPH oxidase induces and increase in ROS release by microglia which was associated with synaptic and cognitive deficits in EAE (Di Filippo et al., 2016) synaptic alterations are likely to occur also with the activity of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and IL-1β released by the microglia (Rossi et al., 2012). Moreover, Microglia is involved in the glutamate uptake but is also one of the important source of glutamate during neuroinflammation (Werner et al., 2001). Thus, overactivation of microglia in MS disease may induce an overproduction of glutamate, as well as increased inhibition of its uptake, compromising the glutamate system homeostasis and excitotoxicity events which may leads to oligodendrocytes death, myelin destruction and neuronal damage (Vercellino et al., 2007; Stojanovic et al., 2014). Moreover, microglial cells mediates synaptic refinement during brain development, a mechanism called synaptic pruning, which was found to be reactivated during the ageing and neurodegenerative disorders such as AD (Rajendran and Policelli., 2018), Likewise, may be involved in MS-associated synaptic loss and cognitive impairment due to the evidences reporting an increase in the key mediators of synaptic pruning process i.e. C3, C1q and CR3 complement components and increased microglia engulfment activity and their colocalization in the post-mortem brain from MS patients and animal models (Stephan et al., 2012; Michailidou et al., 2015). Indeed, C3-KO but

not C1q-KO in EAE mice, reduced disease severity, synapses loss and microglia activity (Hammond et al., 2020)

The physiological regulating role for microglia on basal synaptic transmission and plasticity is also affected during MS. Nisticò et al. studied both inhibitory and excitatory synaptic transmission in the hippocampus EAE mice, reporting a decreasing in the GABAergic signaling and increasing in the glutamatergic transmission associated with a reduced parvalbumin positive cells leading to a significant alterations of synaptic plasticity. In Fact, LTP induction was increases whereas long-term depression (LTD) was inhibited, a deleterious mechanism depending on IL-1β overexpression by overactivated microglial cells (Nisticò et al., 2013). Those results are supported by Parkhurst and colleagues research study in which they investigated the role of microglia in synaptic plasticity and they show that microglia-depleted mice display an abnormal behavioral associated with hippocampal-dependent learning impairment, abnormalities which were associated with abnormal turnover of dendritic spines, and could be mimicked by the lack of brain derived neurotrophic factor (BDNF) (Parkhurst et al., 2013).

1.1.3. Role of the complement system:C1q and C3 activation

The complement system is made up of around sixty circulating and associated proteins membranes that act synergistically and sequentially to perform and regulate their functions. In the immune system, the complement is a part of the innate response which represent the first line of defense against infections through rapid elimination of incoming pathogens and regulation of the response of other immune components. In addition, the complement system eliminates modified self-cells such as apoptotic cells and cellular debris to protect against autoimmunity

(Dunkelberger and Song, 2010). The complement is activated through 3 main pathways: the classic pathway, which is triggered directly by pathogen recognition or indirectly by the complex antibody- surface pathogen antigen (triggered by C1q), the alternative pathway which is continuously activated at a low level as a result of spontaneous C3 hydrolysis and that of lectins, initiated by the binding of mannose-binding lectin (MBL), collectin 11, and ficolins to the pathogen surface (Figure 1.3). All these pathways converge on the activation of the C3 protein to form a key complex known as the C3 convertase. C3 is cleaved into C3a and C3b, opsonization with C3b fragments induces the elimination of structures targeted by phagocytic cells via C3 receptors (CD11b/CD18). The remain C3b can also bind to C4b2b complex resulting from the activation of classical pathways and lectins to form a C4b2b3b complex (i.e. C5 convertase) which generates the C5a anaphylatoxin recognized by the receptor C5aR expressed by phagocytic cells. The C5b then recruits and assembles with the C6, C7, C8 and multiple C9 molecules to form the Membrane attack complex (MAC) which is an important immune effector of the complement terminal pathway with the ability to kill certain microorganisms and to promote inflammation. Both C3a and C5a are known as powerful anaphylatoxins and are effector molecules with diverse functions, including inflammation and modulation of adaptive immunity (Dunkelberger and Song, 2010; Varela and Tomlinson, 2015).

Microglia and astrocytes are the main sources of complement proteins in the CNS, but they are also produced by neurons (Perry and O'Connor, 2010).

The complement plays a key role in the postnatal maturation of brain neuronal circuits. During development, extra synapses are eliminated. This process called synaptic pruning is activity-dependent and it has been first demonstrated in the retinogeniculate system in which less active synapses are engulfed and removed by microglia. Synapse removal is regulated, among other mechanisms, by the classical complement cascade (Druart and Le Magueresse, 2019): C1q and C3 are highly expressed and colocalize with the phagocytic microglia, which are the only cells to express C3R in the CNS, within the weak synapses (stevens et al., 2007). Moreover, C1q- or C3- deficient mice prevented segregation of synaptic inputs into eye-specific regions in the dorsolateral geniculate nucleus (dLGN) resulting in increased densities of excitatory synapses. Furthermore, microglia had reduced phagocytosis of synapses in the dLGN in C3- or CR3-deficient mice (Schafer et al., 2012). Synaptic pruning then was discovered to be mediated in other regions of the CNS such as the spinal cord (Vukojicic et al., 2019) and in the somatosensory cortex (Chu et al., 2010).

The complement system has been implicated in pathological synapse elimination in many psychiatric diseases such as schizophrenia (Hakobyan et al., 2005) and neurodegenerative diseases, especially in AD (Hong et al., 2016). Increased classical complement proteins expression has been found in both acute and chronic MS lesions (Breij et al., 2008; Ingram et al., 2014). The role of complement in MS grey matter degeneration is not fully understood, nevertheless, studies on post-mortem brains from MS patients indicated an early abundant accumulation of the complement components C1q and C3 in the hippocampus in both myelinated and demyelinated C1q and C3 in the hippocampus in both myelinated and

demyelinated regions, which are localized within the microglial processes characterized by increased activity, suggesting that it may be activated a mechanism similar to the developmental synaptic pruning responsible for the observed synapses loss (Michailidou et al., 2015).

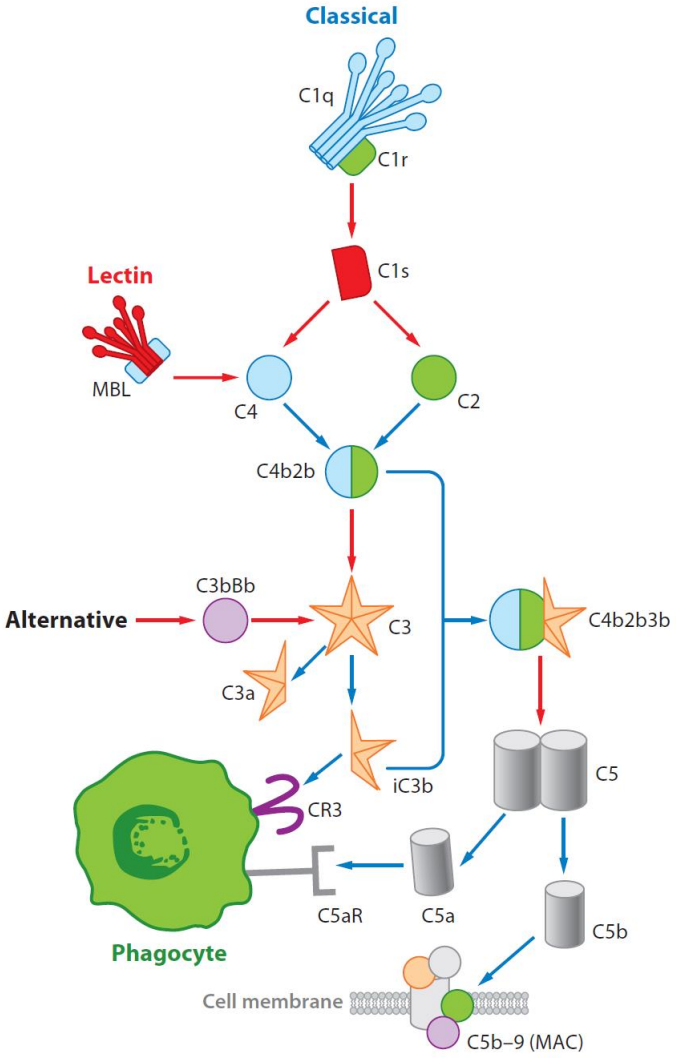


Figure 1.3 The complement cascade activation pathways (Stephan et al., 2012)

In the hippocampus of EAE mice, C1q, C3 levels were elevated and deposited within the synapses. Moreover, the C3 KO mice resulted in less severe disease, reduced microglia activity and protection from the EAE-

induced synaptic loss (Hammond et al., 2020). Several studies have reported the deleterious effects of C3 overexpression in murine models of MS by genetic deletion of C3 (Nataf et al., 2000; Szalai et al., 2007; Smith et al., 2008) or mediating the use of monoclonal antibodies or inhibitors of the C3 convertase (Hu et al., 2012; 2013)

Moreover, Complement terminal pathway product MAC complex are abundant in human white matter lesions, involved in the demyelination process(Rus et al., 2005; Ingram et al.,2014). However, MAC complex have also a protective role toward the oligodendrocytes from apoptotic cell death (Tatomir et al., 2017).

1.1.4. Trem2 as a potential biomarker

Trem2 is an immunoglobulin receptor of the TREM family which is expressed in the CNS by microglial cells under physiological conditions (Schmid et al., 2002). Trem2 or DAP12 loss-of-function mutations cause the well know neurodegenerative disease, Nasu-Hakola, characterized, by demyelination, cerebral atrophy and strong microglial activation. Which highlight the importance of Trem2 and DAP12 in maintaining CNS homeostasis. The mechanisms underlying neurodegeneration of this disease is not yet known but it has been proposed that a defect of function of Trem2 or DAP12 alters the elimination of apoptotic neurons by cells microglia causing accumulation of necrotic debris and harmful compounds. Indeed, The microglial Trem2 has been shown to recognize a Trem2 ligand expressed by apoptotic cells and promoting phagocytosis (Hsieh et al., 2009). In addition, inhibition of microglial expression of Trem2 reduces phagocytosis apoptotic neurons and increases the expression of TNFα and NOS2 (Takahashi et al., 2005). On the other hand, an overexpression of Trem2 increases phagocytosis and decreases the

microglial pro-inflammatory response. Thus, a functional impairment of Trem2 reduces elimination of apoptotic neurons and inflammation which could be the cause of degeneration seen in patients with Nasu-Hakola disease (Gervois et al., 2019). In addition, a loss of function of DAP12 during development has been shown to induce defects in synaptic transmission and plasticity on the glutamatergic synapses in pyramidal neurons from CA1 in the hippocampus to P18-25. One of the consequences of the mutation of DAP12 is to alter the accumulation of TrkB (the BDNF receptor) at postsynaptic densities and modify the effects of BDNF at critical stages of development (Roumier et al., 2004). Thus, Trem2 / DAP12 would facilitate the development and maturation of excitatory synapses.

Less is known about Trem2/DAP12 complex involvement in MS pathophysiology. However, in EAE mice , the expression of Trem2 is increased in the microglia of the spinal cord during the early and chronic phases of inflammation. Inhibition of Trem2 by antibodies directed against this receptor exacerbates disease with an increased inflammatory response (Piccio et al., 2007). Moreover, the transplanted Trem2-overexpressing bone marrow-derived myeloid cells has the protective effect including increasing in the pro-inflammatory molecules, promoting the phagocytosis of myelin debris and reduced damage (Takahashi et al., 2005). In post-mortem brain tissue samples from people with MS, the researchers found high levels of Trem2 expression in active demyelinating lesions. $Trem2^{-/-}$ and $Trem2^{+/-}$ mice showed reduced clearance of myelin debris following treatment with cuprizone (CPZ), a demyelinating model for MS, whereas, treatment with Trem2 agonist promotes microglia phagocytic activity and induces an efficient clearance of myelin debris and promotes benefits effects on axonal regeneration (Cignarella et al., 2019).

Trem2 undergoes proteolytic processing, releasing its ectodomain into the extracellular space as a soluble variant (sTrem2) by ADAM proteases (Wunderlich et al., 2013) and can be detected and measured in human plasma and cerebrospinal fluid (CSF), which levels were found increased in the CSF of MS patients in the RRMS, the SPMS, and the PPMS (Öhrfelt et al., 2016; Piccio et al., 2008) Therefore, the CSF sTrem2 levels might serve as a biomarker for inflammatory diseases including MS (Zhong and Chen., 2019)

1.1.5. Current therapies

To date, there is no effective cure for MS. However, several treatments were developed and approved to improves the daily life of MS patients by minimizing the symptoms such as immunomodulatory medication (Figure 1.4). For example, among the therapies that are actually used to reduce disease progressions we found:

Interferon beta (Avonex): Recombinant forms of IFN are the widely used for MS treatment. They are IFNb-1a and IFNb-1b ; administrated intramuscularly or subcutaneously and subcutaneously respectively. Treatment with IFN drugs reduces the relapse episodes and MRI disease activity (Jacob et al., 1996)

Glatiramer acetate (Copaxone): is a synthetic peptide that has some similarity with the sequence of the MBP which is able to compete with the binding of myelin antigens to the MHC molecules and inhibits the T cell response to several myelin antigens. It has an effective activity in reducing relapse rate in patients with RRMS (Arnon and Sela, 2003).

Natalizumab (Tysabri): is a monoclonal antibody that binds to α4-integrin of the cell adhesion molecule VLA-4, blocking its interaction and binding

to its ligand VCAM1 on the surface of the blood brain barrier (BBB), reducing the relapses by more than 60% and the disability progression (Schreiner et al., 2005).

Dimethyl fumarate (Tecfidera): is an orally administrated immunomodulatory drug that demonstrates a marked reduction and MRI disease activity in patients with RRMS. Its mechanism of action is still not fully understood, but it has been shown to have an anti-oxidant proprieties and reduces the pro-inflammatory T cells including $CD8^+$ and $CD4^+$ T-cell (Linker and Gold, 2013).

Additionally, to physical disabilities, MS patients experience a wide variety of mental disabilities and psychiatric disorders (neuropathic pain, cognitive dysfunction, depression…) that impact negatively their quality life, and are managed especially by some non-pharmacological treatments such as exercising, rehabilitation and psychotherapy (Brenner and Piehl, 2016).

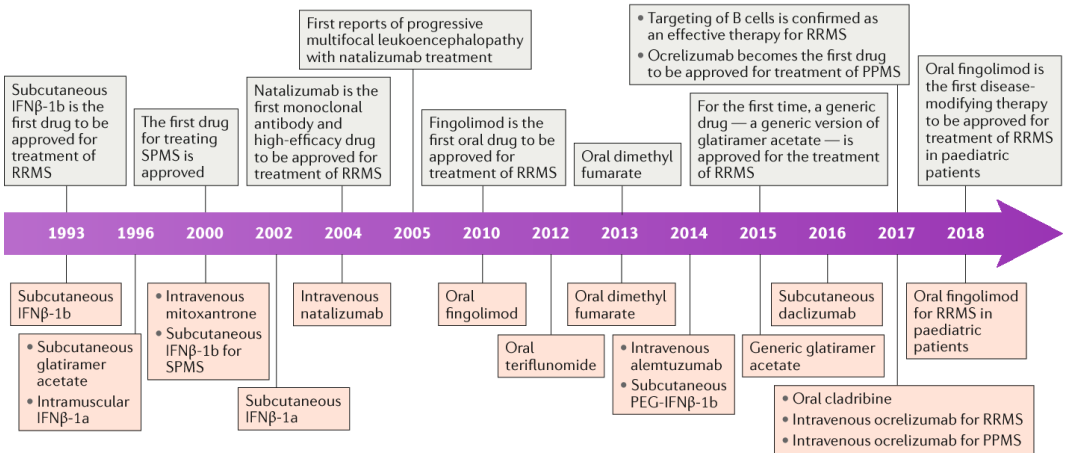


Figure 1.4 Timeline of developments in the treatment of multiple sclerosis (Tintore et al., 2019)

1.2. Animal models of multiple sclerosis

Due to the heterogeneity of MS in its progression, and pathological features, no single animal model is able to fully mimic the disease. Thus multiple animal models have been developed for the investigation of different aspect of the disease including the neuroinflammation and immune system response (EAE in SJL/J or C57BL/6 mice), as well as demyelination, axonal damage and inflammatory response (using Cuprizone, Theiler's murine encephalomyelitis virus, or lysolecithin) (Simmons and al., 2013). However, the most well characterized and commonly used animal model for the study of MS is the experimental autoimmune encephalomyelitis (EAE) model which mimics several pathological aspects of the neuroinflammation, axonal degeneration and demyelination. Most of the current approved therapies for MS was first studies using EAE mice such as glatiramer acetate, mitoxantrone and natalizumab (Croxford et al., 2011).

1.2.1. Experimental Autoimmune Encephalomyelitis (EAE)

The EAE model represents a variety of MS animal models (Poraccini et al., 2015) (Table 1.1). The first model of active EAE was developed by Koritschoner and Schweinburg in 1925 who showed that the injection of a human spinal cord homogenate in rabbits was able to trigger inflammation of the spinal cord with a subsequent paralysis (Stromnes and Goverman, 2006). The analysis of the spinal cord homogenates allowed to understand the importance of myelin antigens on the observed inflammation and demyelination within the CNS. Over the years, The EAE induction protocol was profoundly modified using different antigens, different animal species and the use of novel adjuvant to boost the inflammatory response in the CNS (Kipp, van der Star et al. 2012).

Model	Similarities to human disease	Differences from human disease	Further comments
Lewis rat Active EAE (CNS myelin, MBP, MOG, PLP)	T-cell inflammation and weak antibody response	Monophasic, little demyelination	Reliable model, commonly used for therapy studies. With guinea-pig MBP little demyelination
Adoptive-transfer EAE (MBP, S-100, MOG, GFAP)	Marked T-cell inflammation. Topography of lesions	Monophasic, little demyelination	Homogeneous course, rapid onset. Differential recruitment of T cells/macrophages depending on autoantigen
Active EAE or AT-EAE + co-transfer of anti-MOG antibodies	T-cell inflammation and demyelination	Only transient demyelination	Basic evidence for role of antibodies in demyelination
Congenic Lewis, DA, BN strains Active EAE (recombinant MOG aa 1–125)	Relapsing–remitting disorders, may completely mimic histopathology of multiple sclerosis and subtypes	No spontaneous disease	Chronic disease course, affection of the optic nerve, also axonal damage similar to multiple sclerosis
Murine EAE (SJL, C57BL/6, PL/J, Biozzi ABH) Active EAE (MBP, MOG, PLP and peptides)	Relapsing–remitting (SJL, Biozzi) and chronic-progressive (C57BL/6) disease courses with demyelination and axonal damage	No spontaneous disease	Pertussis (toxin) required for many strains, whilst it is often not needed for SJL and some Biozzi EAE models. Higher variability of disease incidence and course, often cytotoxic demyelination in C57BL/6. With rat MBP inflammatory vasculitis with little demyelination
Murine EAE in transgenic mice or knockout mice (mostly C57BL/6 background)	Specifically addresses role of defined immune molecules/neurotrophic cytokines/ neuroanatomical tracts	Most results obtained with artificial permanent transgenic or knockouts	Extensive backcrossing (>10 times) on C57BL/6 background required. Future work with conditional (cre/loxP) or inducible (e.g. Tet-on) mutants

Table 1.1 The commonly used EAE models (Gold et al., 2006)

1.2.1.1. Induction and disease course

EAE model is based on the stimulation of the peripheral immune system with an emulsion containing Freund's complete adjuvant (CFA) and the myelin peptides such as the MBP or the myelin oligodendrocyte glycoprotein (MOG). The exogenous peptide is recognized and internalized by the resident antigen-presenting cells (APCs) at the site injection. These, migrate to the draining lymph nodes to activate T helper (TH) naïve cells (TH1 and TH17) via MHC class II, which, are subsequently able to reach the CNS and recognize the homologs endogenously antigen present in the animal's CNS where they are then reactivated by non-professional APCs present in the CNS (for example, microglia). The T effector cells then undergo clonal expansion and participate in the recruitment and activation of more peripheral immune

cells (effector T cells, B cells, macrophages, etc.) (Steinman, 1996; Becher et al., 2006)

In our model, C57BL/6 mice are induced by subcutaneous injection of MOG antigen emulsified in CFA, which contains a combination of mineral oil and Mycobacterium tuberculosis strain H37Ra that is highly immunogenic, the immunization is accompanied by intraperitoneal injections of pertussis toxin (PTX) . Most often two injection points are realized. Then, an additional intraperitoneal injection of PTX, is done 24 hours after the primary injection (Bittner et al. 2014). The resulting disease course is the chronic-progressive form of MS without the episode of remission. The disease progression is characterized by an ascending paralysis, beginning at the tail to the forelimbs which can be scored using a well-established body condition score that reflect increasing levels of paralysis.

EAE may be also induced in mice by adoptively transferring peripheral autoreactive lymphocytes from MOG-immunized animals to naïve animals (Hohlfeld and Steinman, 2017).

The course of EAE depends disease on the immunization protocol, myelin antigen and adjuvant used, as well as on the species, strain, gender and age of the animals used to induce the disease. For example, Immunization of SJL/J mice with the encephalitogenic PLP139–151 peptide induces a relapsing remitting EAE in SJL mice, whereas MOG35-55 triggers a chronic progressive EAE in C57BL/6 mice, depending on the aspect of MS to study. Though, inflammation and demyelination are present in almost all EAE mice (Poraccini et al., 2015)

1.2.1.2. Limitations

The EAE model has proven to be remarkably valuable in MS research. Most of preclinical studies of the approved treatment for MS are done using the EAE model to determine their efficacy and preliminary safety. Furthermore, EAE is a valuable model to study the pathogenesis of the disease such as the inflammatory mechanism and the disruption of the BBB, nevertheless, to discover new pathogenic pathways. Interestingly, in MS patients and EAE animals was observed similar characteristic, including disseminating lesions, myelin damage, axonal degeneration and neuronal loss. Additionally, MS and EAE share most of the immune components involved in the disease onset and progression including CD4+ and CD8+ T-cells with same reactivity to the myelin antigens (Steinman and Zamvil, 2005; 2006)

There are, however, differences and limitation of this model compared to MS. The most important, is the timing of the disease onset and the limitation of the progression i.e. EAE disease onset few days after the immunization whereas MS development it may take months or years before the first symptoms appear (Constantinescu et al., 2011). In addition, studies in EAE are time-limited i.e. EAE experiments, usually, last after a short period of time (two to three weeks for the acute stage and few months to study the chronic stage) (Steinman and Zamvil, 2005) which does not represent to the real MS, therefore, it may not be possible to use the model to study accurately the long term disease course of MS.

1.3. Hippocampus pathology in multiple sclerosis

1.3.1. The hippocampus

The Hippocampus, is a region located within the temporal lobe of the brain, forms part of the limbic system, that is well known to be associated primarily with spatial navigation and memory formation (Anand and Dhikav, 2012).

The hippocampus receives afferent inputs from different regions of the brain and the limbic system and has various connections to other cortical and subcortical regions. It can be distinguished in anatomic subregions with distinct cytoarchitecture and connections that form two major regions: the dentate gyrus (DG) and cornu ammonis (CA) fields (CA1–CA3) which wrap around each other and interconnected by layers consisting of axons, dendrites and different type of cell bodies (Deykeyzer et al. 2017). The internal part of the hippocampus is distinguished into layers that include the alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare and the stratum lucidum. The stratum pyramidale forms three subfields of pyramidal neuron cell bodies (glutamatergic neurons) with different characteristics, the CA1, CA2 and the CA3. The apical dendrites of the pyramidal neurons extend into the hippocampus, forming the stratum radiatum, in this layer, synapses occur with Schaffer collaterals pathway (Knierim 2015) (Figure 1.5).

The stratum lacunosum-moleculare is important because it serves as a relay between the entorhinal cortex (EC) and the CA1, is formed by dendrites of pyramidal neurons, and especially by the terminals of fibers originating from the EC and GABAergic interneurons (Capogna, 2011).

The dentate gyrus can be divided into three layers: the stratum moleculare, containing granule cell dendrites; the stratum granulosum,

containing the granule cell bodies (excitatory neurons); and the polymorphic layer, contains mossy fibers. The polymorphic and molecular layers of the dentate gyrus contain basket interneurons, which are important inhibitors of granule cells (Amaral et al., 2007)

The hippocampus has three major intrinsic unidirectional pathways (Figure 1.5) that form the trisynaptic circuit. The first consists of the perforating pathway that connects the entorhinal cortex to the dentate gyrus, this projection constitutes the source of the hippocampal afferents. The second unidirectional projection is formed by the axons of the granular cell bodies of the dentate gyrus which project only to CA3 via mossy cell fibers. The CA3 then forms connections to CA 1 via Schaffer collaterals which form the third unidirectional path. Finally, the main efferent pathway of the hippocampus emerges from CA1 to reach the subiculum (Knierim, 2015). In conclusion, the entorhinal cortex is considered to be the "gateway" main information to the hippocampus while the subiculum occupies the role of "Exit gate" because it is considered the major efferent pathway of the hippocampus (Mu and Gage, 2011). Each region within the trisynaptic circuit is crucial for their unique contribution to the overall mnemonic processes conducted by the hippocampus.

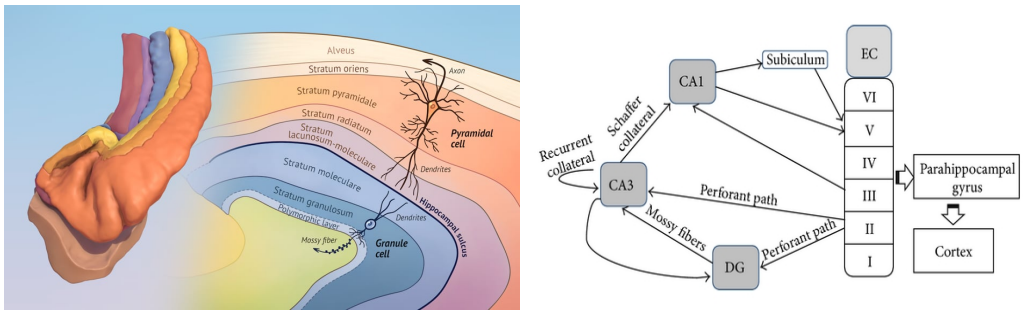


Figure 1.5 Adult human hippocampal formation and trisynaptic circuit (Yau and So, 2015; Brandt et al. 2019)

The case of Henry G. Molaison (H.M.) is probably the best known single patient in the history of neuroscience. H.M is an epileptic patient whose was unable to form new declarative memories following a bilateral surgical ablation of medial temporal lobe structures, including the hippocampus. This suggested that the hippocampus plays an important role in memory formation and consolidation, but that other structures are involved in the long-term storage of this memory (Annese et al., 2014). Moreover, multiple studies in rodent, have shown how dorsal hippocampal lesions markedly impaired spatial recognition (Moser et al., 1993; 1995; Duva et al., 2007), thus suggesting that the hippocampus is necessary region in the spatial memorization memory.

1.3.2. Synaptic plasticity

Long term plasticity: The formation of new memories requires changes in exiting neural connections and/or the formation of new connections in the brain. The neuronal ability to change their connections over the time, depending on the sensory experience, the development and experience of an individual is called neuroplasticity (Schaffer, 2016). The synaptic plasticity, is referred the changes that occur at the level of the synapses requiring both presynaptic and postsynaptic activity (Ramirez and Arbuckle, 2016).

LTP is a persistent synaptic strengthening process, defined by a increased postsynaptic response following strong presynaptic activation. This potentiation can be reversed by other stimulation protocols, called depression (long term depression; LTD). Thus, changes in the efficiency of the synaptic connection are bidirectional (Barr et al., 1995).

LTP is the most studied form of synaptic plasticity, and the one that, in mammals, that widely accepted to represent the general mechanism of memory formation in the hippocampus (Bliss and Lømo, 1973).

LTP has been most thoroughly studied and induced in the CA3-CA1 Schaffer collateral synapses of the hippocampus by bursts of electrical stimuli. Along the CA3-CA1 connections, there is a release of glutamate presynaptically, which activate the postsynaptic glutamate receptors (iGluRs), The most commonly referenced iGluRs are N-Methyl-D-aspartate (NMDA) and α-amino-3- hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) (Malenka and Nicoll 1999). Based on the dependence of gene transcription and protein translation, long-term potentiation (LTP) can be divided into two phases early-phase LTP (E-LTP) (lasting 1–2 h) and late-phase LTP (L-LTP) (lasting >3 h) . For the early phase of LTP (E-LTP), it first requires the activation of pre-existing membrane AMPA receptors, which provides the majority of inward current for generating synaptic responses. After the depolarization of the postsynaptic cell, NMDA receptors are activated and Mg2+ dissociates from its binding site, allowing Ca2+ as well as Na+ entering. The rapid rise in intracellular Ca2+ concentration triggers the activation of several enzymes that mediate early-phase LTP. Among these, calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), PKA and mitogen-activated protein kinase (MAPK) play an important role in LTP (Malenka and Nicoll 1999). For the L-LTP, it requires new synthesis in the postsynaptic proteins involved in the mechanism of LTP. The transition between early-phase LTP and late-phase LTP is be mediated particularly by the activation of the extracellular signal-regulated kinase (ERK) (Adam and Dudek, 2005).

Sustained activation of synapses increases in strength resulting in LTP reaching the maximum response which it may inhibit the formation of new connection to encode for new incoming information. Therefore, compensatory mechanism is needed and which is known as LTD, this process is activity- dependent and mediates the synaptic transmission weakening. Whereas LTP at these synapses requires brief, high-frequency stimulation at short time, LTD occurs when the Schaffer collaterals are stimulated at a low frequency stimulation for longer periods. Similar to LTP, LTD requires activation of NMDA receptors and the resulting entry of calcium in the postsynaptic cells (Graham et al., 2010; Wiegert and Oertner, 2013).

Short term plasticity (STP): refers to a phenomenon in which synaptic efficacy changes last on the order of milliseconds to several minutes. This form of plasticity is believed to be important in mediating the fast adaptations to transient sensorial changes and short-lasting forms of memory, which in vitro obtained by short bursts of electrical stimulus causing a transient accumulation of calcium in presynaptic nerve terminals resulting in transient changes in the probability of presynaptic neurotransmitter release (Zucker and Regehr, 2002).

Two forms of STP, with opposite effects on synaptic efficacy, have been largely studies, The short term facilitation (STF) and the short term depression (STD) (Zengel et al., 1980; Zucker and Regehr, 2002). To study this form of plasticity, two stimuli must be delivered within a short interval, the response to the second stimulus can be either increased or depressed compared to the response to the first stimulus, which apparently depends on the residual availability of presynaptic internal [Ca^{2+}] resulting in paired-pulse depression (PPD) in case of a transient

depletion of neurotransmitter vesicles at the presynaptic terminal or paired-pulse facilitation (PPF) even at longer interstimulus intervals (20–500 ms) due to the residual calcium left over from the first stimulus which contributes to additional release during the second stimulation. Because these forms of plasticity largely result from changes in the probability of transmitter release, Synapses that have a low probability of release tend to display large facilitation. In contrast, synapses with high probability of release tend to display depression (Malenka and Citri, 2008).

1.3.3. Hippocampus synaptic dysfunction in multiple sclerosis

40 to 70% of the MS patients suffer from cognitive deficits including memory impairment. The hippocampus is one of the brain region particularly sensitive to the detrimental effects of neuroinflammation, indeed, it has been implicated in several inflammatory neurodegenerative diseases such as AD and PD (Mancini et al., 2017; Kinney et al., 2018; Lim et al., 2018). Moreover, the hippocampus is important for learning and memory process, and is vulnerable to the deleterious effect of MS pathology (Mancini et al., 2017), which makes it one of the most critical involved in the memory impairment experienced by MS patients.

To date, what is known about the involvement of the hippocampus in the pathophysiology of MS disease is derived from histological studies in post-mortem MS patient's brains and from animal models especially by using the EAE models. Several and controversial studies were aimed at the study the effect of MS on the hippocampal synaptic plasticity in the EAE mice, in our laboratory we have analyzed the most studied long term synaptic plasticity, LTP and LTD, in the CA1 region, we have shown a significant increase in LTP and inhibition in LTD induction which were attributed it to an increase in the release of IL- 1β from the activated

microglia, indeed, the perfusion of IL- 1β in hippocampal slices from untreated mice have shown the same electrophysiological results. Moreover, GABAergic and glutamatergic synaptic transmission ratio was analyzed, decreased in the inhibitory responses was correlated to the loss of parvalbumin positive cells in the CA1 layer, no change was found in the glutamatergic transmission (Nisticò et al., 2013). Studying the synaptic plasticity and memory-dependent behaviors in the EAE mice during the peak and the acute stage of the disease, Kim do et al., have found hippocampal atrophy, increasing in the pro-inflammatory cytokines such as IL-1β and TNF-α, and inhibition in the LTP process in the CA1 region associated with an impairment in the spatial learning and memory deficits (Kim et al., 2012). In addition, in vivo electrophysiological analysis in the EAE rat have demonstrated a suppressed LTP induction which may be related to an increase of inhibitory control of the GABAergic neurons in the CA1 region (Mosayebi et al., 2016). Novkovic and colleague have demonstrated a stage-dependent alteration in the LTP induction, LTP was unaltered in the acute stage but impaired in the late progressive phase of the disease (Novkovic et al., 2015). Additional evidences have confirmed the increase in microglia activity, proinflammatory cytokine release and impairments in LTP in the hippocampus of the EAE model (Di Filippo et al., 2013; 2016).

Studies in the post-mortem brains and MRI analysis confirm what have been reported in the MS models. Demyelination, decreased in synaptic density, hippocampal atrophy was found (Dutta et al., 2011; Michailidou et al., 2015) and were associated with cognitive impairments (Mandolesi et al., 2015; Preziosa et al., 2016). Hippocampal synaptic loss may be the results of the activation of a mechanism similar to the developmental

synaptic pruning, indeed, an increase in the C1q and C3 complement proteins and microglia activity was reported in the MS post-mortem brains and in the EAE mice (Michailidou et al., 2015; Nack et al., 2019; Hammond et al., 2020).

Moreover, several MS patients had episodes of memory and cognitive impairments in the early stage of the disease (McNicholas et al., 2017), therefore, It has been suggested that it may be and early hippocampal synaptic impairment (Mancini et al., 2017). Indeed, spatial memory and cognitive impairments in EAE mice were observed even before the motor symptoms onset (Kim et al., 2012; Acharjee et al., 2013) which highlight the hypothesis of a possible early hippocampal structural and functional alteration.

2. Aims and objectives

In addition to motor/sensory deficits, there are cognitive difficulties in the early stage of the disease in MS patients and animal models, even before motor symptoms are present (Kim et al., 2012; Acharjee et al., 2013; McNicholas et al., 2017). Although hippocampal involvement in the pathophysiology of memory impairment in MS disease has been widely investigated, results are controversial and very little is known about its involvement in the asymptomatic phase, before clinical symptoms onset. Furthermore, preclinical evidences suggest that classical complement proteins and microglia may drive cell, axon and myelin damage as reported in the hippocampus of MS patients (Ingram et al., 2014; Michailidou et al., 2015).

Therefore, the hypothesis is that hippocampal synaptic loss and dysfunction might lead to progressive memory impairment. Therefore, we investigated the early asymptomatic stage of the disease, focusing on the role of microglia cells and related signaling pathways. We believe that overactivation of these cells might contribute to the early synaptic alterations which lead to disease progression and clinical manifestation in the EAE model.

Studying the asymptomatic stage of MS disease may be useful because it may help to find novel biomarkers as well as to identify potential targets for disease-modifying therapeutic intervention.

The central aim of this thesis is to investigate the early synaptic dysfunction in the hippocampus of EAE mice, to identify the link between microglia hyperactivity and hippocampal synaptic dysfunction in the asymptomatic stage of EAE, and to understand the role of the major

player of microglia phagocytic activity, C3, C3R and Trem2 on EAE clinical onset and progression.

The objective of this thesis is to answer to the following questions:

1) Is glutamatergic and GABAergic synaptic density reduced, increased or unchanged in the asymptomatic stage of EAE?
2) Does microglia overactivation in EAE lead to the early hippocampal synaptic loss?
3) Is there any alteration in the hippocampus synaptic transmission and plasticity in the asymptomatic stage of EAE?
4) Are the complement protein C3 and microglial phagocytic receptors C3R and Trem2 involved in the EAE progression?
5) Does the targeting of complement protein C3 and microglial phagocytic receptors C3R and Trem2 rescue the EAE progression?

3. Methods

3.1. Mice

All experiments and procedures were approved by The Centre for Phenogenomics (TCP) animal care committee and conformed to the Canadian Council on Animal Care (CCAC) guidelines. All efforts were made to minimize animal suffering and to reduce the number of mice used. For all experiments, female mice were 9-12 weeks of age. All KO mice were purchased from Jackson Laboratories and bred with C57BL/6J colony in TCP; Homozygous Trem2$^{-/-}$ (B6; Trem2em2Adiuj/J), C3$^{-/-}$ mice (B6;129S4-C3tm1Crr/J) and CD11b$^{-/-}$ (B6; 129S4-Itgam^{tm1Myd}/J). Mice were housed 4 per cage in a controlled environment maintained on a 12:12 hour light:dark (7AM lights on,7PM lights off) and allowed ad libitum access to food and water and controlled (22–23°C) temperature.

3.2. EAE induction

EAE was induced in female mice 9-12week old, by subcutaneous (in the upper and lower back) immunization with 200 μL of pre-mixed emulsion (Hooke Labs EK-2110) containing 200 μg myelin oligodendrocyte glycoprotein, peptide 35–55 (MOG 35−55) in incomplete Freund's adjuvant (CFA) containing 8 mg·mL^{-1} heat-inactivated Mycobacterium tuberculosis (strain H37Ra). *Pertussis* toxin (Hooke labs) (100 ng) was injected intraperitoneally (i.p) (100 μL) on the day of the immunization and again 24 hours later. Body weight and clinical score (0 = healthy; 1 = limp tail; 2 = weakness of hindlimbs; 3 = paralysis of hindlimbs; 4 = quadriplegia; 5 = death due to the EAE) were recorded daily. For all mice, the score was evaluated in blind. Control sex-matched mice were injected

with control emulsion without MOG35-55 (Hooke Labs CK-21100) and Pertussis toxin, referred as CFA.

3.4. Immunohistochemistry

3.4.1. Brain tissue processing

Brains tissue from EAE and CFA mice were processed on the asymptomatic stage of the disease, before the motor symptoms appear. At day 4 and day 8 post-immunization (4 d.p.i and 8 d.p.i). Brains were rapidly extracted and then were fixed by immersion in 10 mL of 4% PFA in 1XD-PBS prepared fresh from 16 % stock (EM Sciences, Cat no. 15710) and fixed for 23 hours at 4°C. Subsequently, brains were washed three times in 1X D-PBS and then dehydrated in 20 mL of cold (4°C), 30 % sucrose in 1XD-PBS (w/v), and kept at 4°C for 72 hours. Finally, brains were embedded in optimal cutting temperature (OCT) medium (Tissue Tek, Cat no. 4583), flash frozen on liquid nitrogen for 2 minutes and stored at -80° C until sectioning. All mice were dissected with experimenter blinded to their treatment and all KO were dissected with experimenter blinded to their genotype.

3.4.2. Primary and secondary antibodies staining

Sagittal sections from the embedded brains were cryo-sectioned at 25 μm and mounted onto charged slides, in the experimental day. Mounted slides were washed 3 times in D-PBS to remove sectioning medium and were dried at room temperature for 10-15 minutes. Sections, then were blocked and permeabilized in 0.1% Triton X-100 in 1X D-PBS with 5% normal donkey serum (NDS; Jackson Immuno.Research, cat. no. 017-000-001) for 40 minutes at room temperature. Next, sections were stained with primary antibodies in 0.1% Tween-20 in 1XD-PBS and kept overnight (17-

19 hours) at 4°C. Sections were then washed three times in 1XD-PBS for 5 minutes and next stained with Alexa fluorophore-conjugated secondary antibodies (Alexa 488, Cy3 or Cy5) and DAPI (SigmaAldrich, cat. no. D8417) in 1XD-PBS for two hours at room temperature, placed a horizontal shaker. Next, Sections were washed three times in 1XD-PBS for 5 minutes and mounted with 80 µL of ProLong Glass (Invitrogen, cat. no. P36980) and no. 1.5 high precision coverslips (Thorlabs, cat. no. CG15KH1). List of all the primary and secondary antibodies used in this thesis are listed in Table 1.2.

Primary antibodies	Animal host	Dilutions	Source (code)	Secondary antibodies	Dilutions	Source (code)
Anti-Iba1	Rabbit	1/1000	Wako 019-19741	Donkey anti-Rabbit	1/1000	Jacksonimmuno. (711-545-152)
Anti-CD68	Rat	1/500	Bio-rad (MCA1957)	Donkey anti-Rat	1/1000	Jacksonimmuno. (712-175-153)
Anti-CD11b	Rat	1/250	Bio-rad (MCA 711)	Donkey anti-Rat	1/1000	Jacksonimmuno. (712-175-153)
Anti-C1q	Rabbit	1/500	Abcam (ab182451)	Donkey anti-Rabbit	1/1000	Jacksonimmuno. (711-545-152)
Anti-MBP	Rat	1/250	Millipore (MAB386)	Donkey anti-Rat	1/1000	Jacksonimmuno. (712-175-153)
Anti-VGAT	Rabbit	1/500	Synaptic systems (131003)	Donkey anti-Rabbit	1/1000	Jacksonimmuno. (711-545-152)
Anti-Gephyrin	Rabbit	1/500	Synaptic systems (147 008)	Donkey anti-Rabbit	1/1000	Jacksonimmuno. (711-545-152)
Anti-Vglut1	Guinea pig	1/500	Synaptic systems (ab5905)	Donkey anti-Guinea pig	1/800	Jacksonimmuno. (706-545-148)
Anti-Vglut2	Guinea pig	1/500	Synaptic systems (135404)	Donkey anti-Guinea pig	1/800	Jacksonimmuno. (706-545-148)
Anti-Homer1	Rabbit	1/1000	Synaptic Systems (131 003)	Donkey anti-Rabbit	1/1000	Jacksonimmuno. (711-545-152)

Table 1.2 List of primary and secondary antibodies

3.5. Confocal microscopy and quantification

3-4 immuno-stained tissues per animal were analyzed. Mounted slides were examined under a confocal microscope (Nikon A1R HD25) using 60X oil objective and 2x and 8x zoom at 1024x1024 pixels. 2 field of view were acquired for all images and 4 μm z-stacks were acquired (z-step = 0.25 μm) for each hippocampus from each section. Confocal acquisition setting, laser power and detector gain, was kept constant through the acquisition and for each single analyzed marker. For each section, the CA1-stratum radiatum, the CA1-stratum lacunosum and the DG-molecular layer hippocampal areas were imaged and in blind to the treatment, images analyses were done using Nikon NIS Elements, the threshold was set manually and giving a value of 0 for secondary controls antibodies. All measurements values were averaged values from 3-4 image stacks for 2 field of view and for each animal and expressed relative to CFA controls and graphed as mean ± SEM.

3.6. Electrophysiology

3.6.1. Acute hippocampal slice preparation

To measure the basal synaptic transmission and synaptic plasticity in the CA1 and DG, electrical protocols stimulation and excitatory postsynaptic potential field (fEPSP) recordings were performed on acute hippocampal slices. Mice from EAE and CFA groups were anaesthetized with isoflurane and killed at 4d.p.i and 8d.p.i (score=0). Brains were quickly removed and placed in ice-cold cutting solution containing (in mM): 85 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 10 D-glucose, 75 sucrose). Next, the hippocampi were dissected and 400 μm-thick sagittal slices were prepared on a vibratome (Leica VT1200S). Slices are left to recover

submerged in an incubation chamber at room temperature for 2-3 hours in artificial cerebrospinal fluid (ACSF) saturated with 95% O_2/5% CO_2. ACSF composition (all purchased from MULTICELL): 26 mM NaHCO3, 10 mM glucose, 124 mM NaCl, 1.25 mM NaH2PO4, 3 mM KCl, 1 mM MgCl2 and 2 mM CaCl2; pH was adjusted to 7.4, osmolarity to ~300 mOsm.

3.6.2. Extracellular field recording

After the recovery period, hippocampal slices were transferred to the recording chamber continuously perfused with oxygenated ACSF at 30° C at a flow rate of 2.5 mL/min. Stimulating bipolar matrix Pt;Ir microelectrode (FHC, Inc., CA, USA) and borosilicate filamented glass recording electrodes (2MΩ) recordings (World Precision Instruments) were filled with ACSF and were placed along the Schaffer collateral (SC) pathway or the medial perforant path (MPP). Signals were amplified using axopatch 1D amplifier (Axon instruments) set to 5 kHz low-pass filtering and digitized at 20 kHz using a Digidata 1200 and pCLAMP8 software. Responses were electrically evoked by pulses spaced 40 ms apart at a constant intensity (0.1 ms pulse width) throughout the experiments. Responses were evoked and collected every 20 s and the slope of evoked fEPSPs (V/s), 30% of the maximum response, was measured. Input/output (I/O) data were obtained by recording responses with 0.5, 1, 2, 3, 5, mA stimulation. Short-term plasticity was assessed by recording responses to paired pulses delivered 50, 100, 150, 200, 250, and 500 ms apart. Recordings were monitored and analyzed using WinLTP. Each experiment was conducted on slices from separate animals, therefore the n value represents both the number of slices and animals used.

CA1-SC LTP: Following a stable baseline period of 30 minutes, LTP was induced using compressed theta-burst stimulation (cTBS) protocol, delivered at the same basal stimulus intensity. three episodes of TBS include 5 bursts at 5 Hz, each burst is composed of 5 pulses at 100 Hz, for each episode delivered with an inter-episode interval of 10 s (Park et al., 2019).

DG-MPP LTP*:* Following a stable baseline of 30 minutes, tetanic stimulation was applied for LTP induction in the presence of 10 μM bicuculline (HelloBio) to inhibit the GABAergic transmission. LTP was induced with 100 Hz tetanus (0.15 ms pulses delivered in 4 trains of 0.5 s duration; trains spaced 20 s apart) (Saab et al., 2009).

3.7. Statistical analysis

All data was graphed and statistically analyzed using GraphPad Prism software, significance was defined as $p < 0.05$. Unpaired two-tailed t-tests and Pearson correlation tests were used to assess differences and variance among EAE and CFA groups, N values reflect the number of animals. ANOVAs multiple comparisons test were used for analysis of the clinical scores of differences between EAE KO mice and their EAE littermates. For LTP experiments, all data were normalized to baseline and for the comparison of the magnitude of LTP, the last 10 min of recordings were compared statistically, using unpaired two-tailed Student's t test. All data are expressed as the mean ± standard error of the mean (SEM).

4. Results

4.1. Characterization of microglia activity in the asymptomatic stage of EAE

To test whether microglial activation is increased in the hippocampus of asymptomatic EAE mice, we performed immunohistofluorescence on brains from EAE mice and compared it to CFA controls at days 4 and 8 post-immunization, when motor symptoms were not present; clinical score=0. C57BL/6J female mice (9-12 weeks old) were immunized with MOG35-55 and scored daily for EAE progression. Clinical signs of motor abnormalities start to be observed between 9 d.p.i and 11 d.p.i, whereas, CFA female mice at the same age have showed no clinical signs for the entire duration of the experiment (35 days) (Figure 1.6).

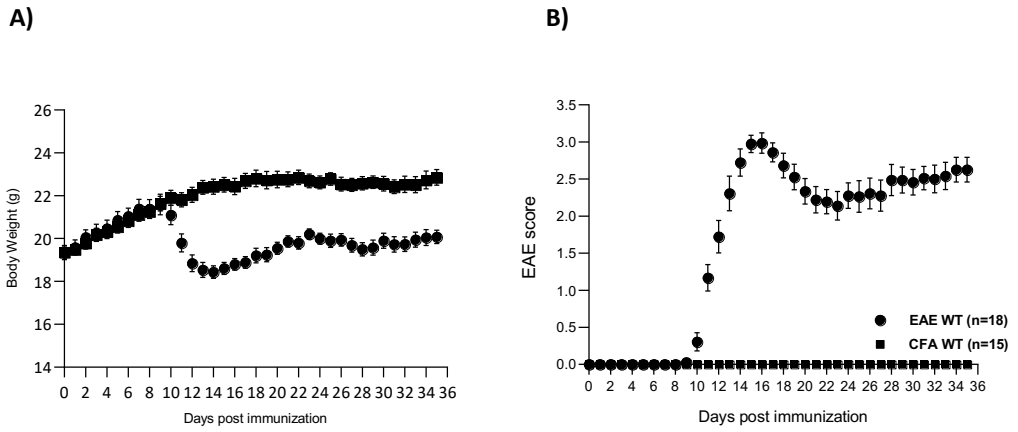


Figure 1.6 C57BL/6J wild type (WT) EAE mice show no motor deficits prior to day 9 post-immunization. Daily monitored (A) body weight and (B) clinical score in the EAE (N=18) and CFA (N=15) mice for 35 days; the EAE mice developed motor signs between 9 and 11 d.p.i reaching the peak of the clinical severity on 15-16 d.p.i, mice then show a period of motor symptoms improvement (20-28 d.p.i) followed by a chronic exacerbation in clinical score (28-35 d.p.i).

As the hippocampus most critical pathways are the perforant pathway (PP): is the source of the major input , and the SC pathway: the source of the principal output (Mu and Gage, 2011), we chose to focus all the study of the thesis on the CA1 and DG areas of the hippocampus (Figure 1.7).

4.1.1. Iba-1 and CD68 immunostaining show unaltered microglia activity in the EAE hippocampus on 4 d.p.i and 8 d.p.i

To investigate the microglia activation state in the hippocampus of EAE mice at 4 and 8 d.p.i compared to CFA controls, we began by the evaluation of immunoreactivity of the ionized calcium-binding adaptor molecule 1 (Iba-1), constitutively expressed by microglia cells in the brain and the most used immunohistochemical marker (Hopperton et al., 2018; Waller et al., 2019), and the microglial lysosomes marker CD68, widely used as a marker for microglia activation (Hopperton et al., 2018; Werneburg et al., 2020), in the CA1 stratum radiatum (CA1-str.R) (Figure 1.8 A and B), in the CA1 stratum lacunosum moleculare (CA1-SLM) (Figure 1.8 C and D) and in the dentate gyrus molecular layer (DG-ML) (Figure 1.8 E and F).

At 4 d.p.i and 8 d.p.i, there was no significant difference between EAE and CFA mice in Iba-1 expression levels or in Iba-1 positive cells, in the CA1-str.R (4 d.p.i, Figure 1.8 A and Ai; p=0.47, p=0.1 respectively. 8 d.p.i, Figure 1.8 B and Bi; p= 0.87, p=0.72 respectively), in the CA1-SLM (4 d.p.i, Figure 1.8 C and Ci; p=0.4, p=0.9 respectively. 8 d.p.i, Figure 1.8 D and Di; p=0.9, p=0.8 respectively) and in the DG-ML (4 d.p.i, Figure 1.8 E and Ei; p= 0.64, p=0.98 respectively. 8 d.p.i, Figure 1.8 F and Fi; p= 0.87, p=0.36 respectively). Therefore, there was no change in the level of microglial activation between the two groups; CD68 immunoreactivity and $CD68^{+}$ cells show no

significant difference in the CA1-str.R (4 d.p.i, Figure 1.8 A and Ai; p=0.54, p=0.06 respectively. 8 d.p.i, Figure 1.8 B and Bi; p= 0.36, p=0.81 respectively), in the CA1-SLM (4 d.p.i, Figure 1.8 C and Ci; p=0.15, p=0.09 respectively. 8 d.p.i, Figure 1.8 D and Di; p=0.4, p=0.95 respectively)and in the DG-ML (4 d.p.i, Figure 1.8 E and Ei; p= 0.63, p=0.19 respectively. 8 d.p.i, Figure 1.8 F and Fi; p= 0.56, p=0.0 respectively). In addition, we examined if there was an increase in the co-localization between Iba-1 and CD68 expressing cells by analyzing the Iba-1^{+}/CD68^{+} cell volume, results show no significant difference at 4 d.p.i and 8 d.p.i in all the three analyzed hippocampal area; CA1-str.R (4 d.p.i, Figure 1.8 A and Ai; p=0.31. 8 d.p.i, Figure 1.8 B and Bi; p= 0.88), CA1-SLM (4 d.p.i, Figure 1.8 C and Ci; p=0.51. 8 d.p.i, Figure 1.8 D and Di; p= 0.91)and DG-ML (4 d.p.i, Figure 1.8 E and Ei; p=0.28. 8 d.p.i, Figure 1.8 F and Fi; p= 0.9).

Similar to previous findings (Acharjee et al., 2013), these data confirm that in the hippocampus of EAE mice during the asymptomatic phase, and even at 8 d.p.i which is the day before the disease onset, there was no abnormal microglia activity in the hippocampus of EAE mice compared to their CFA controls.

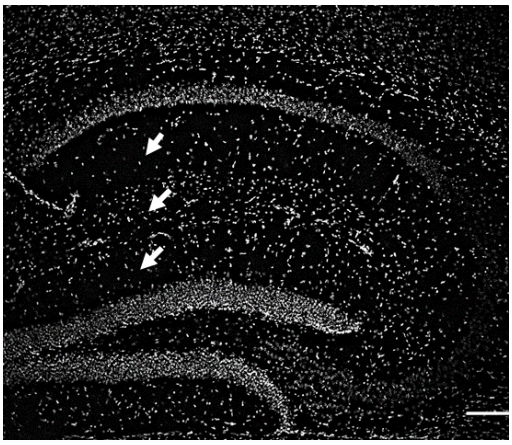
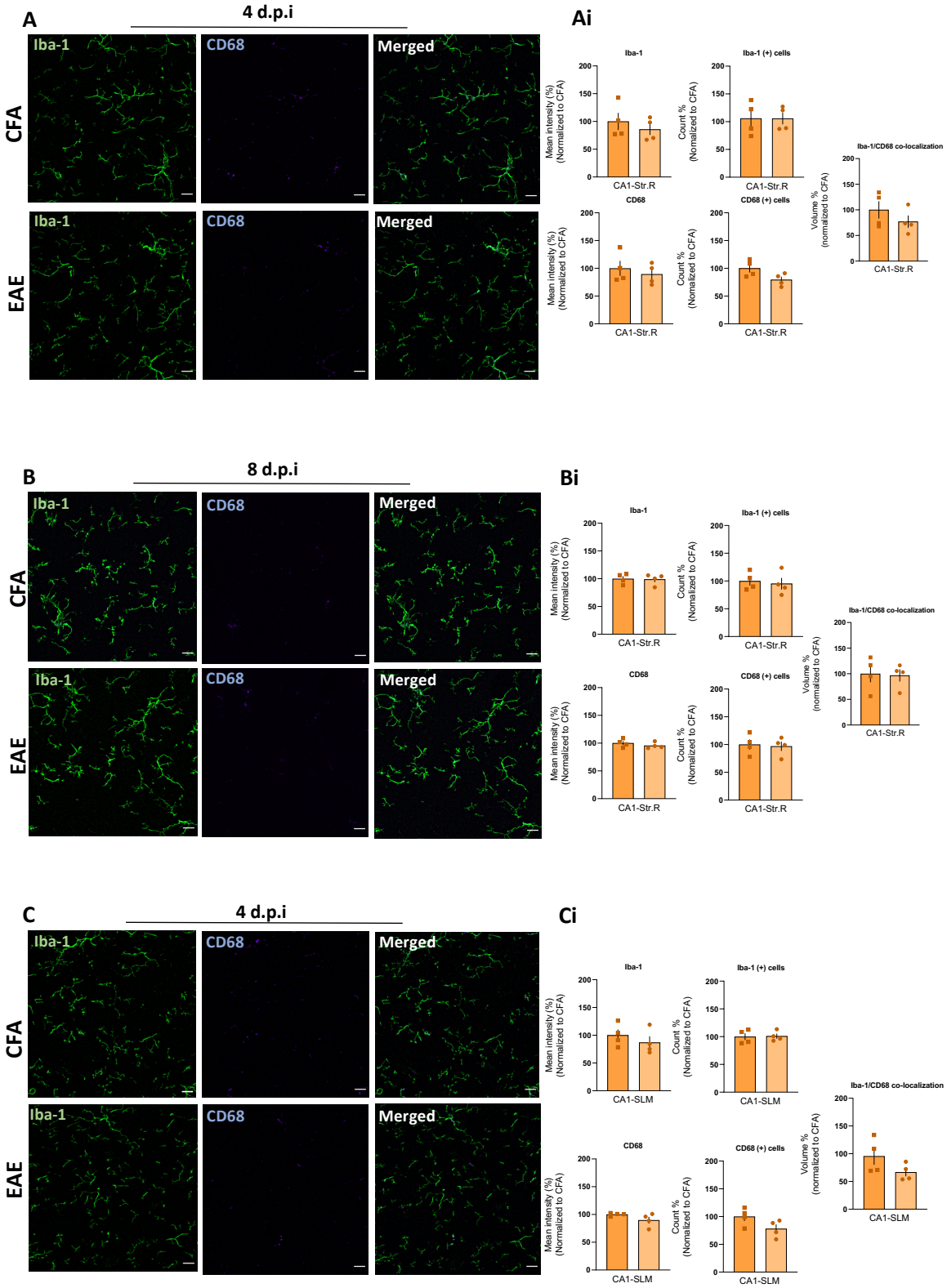
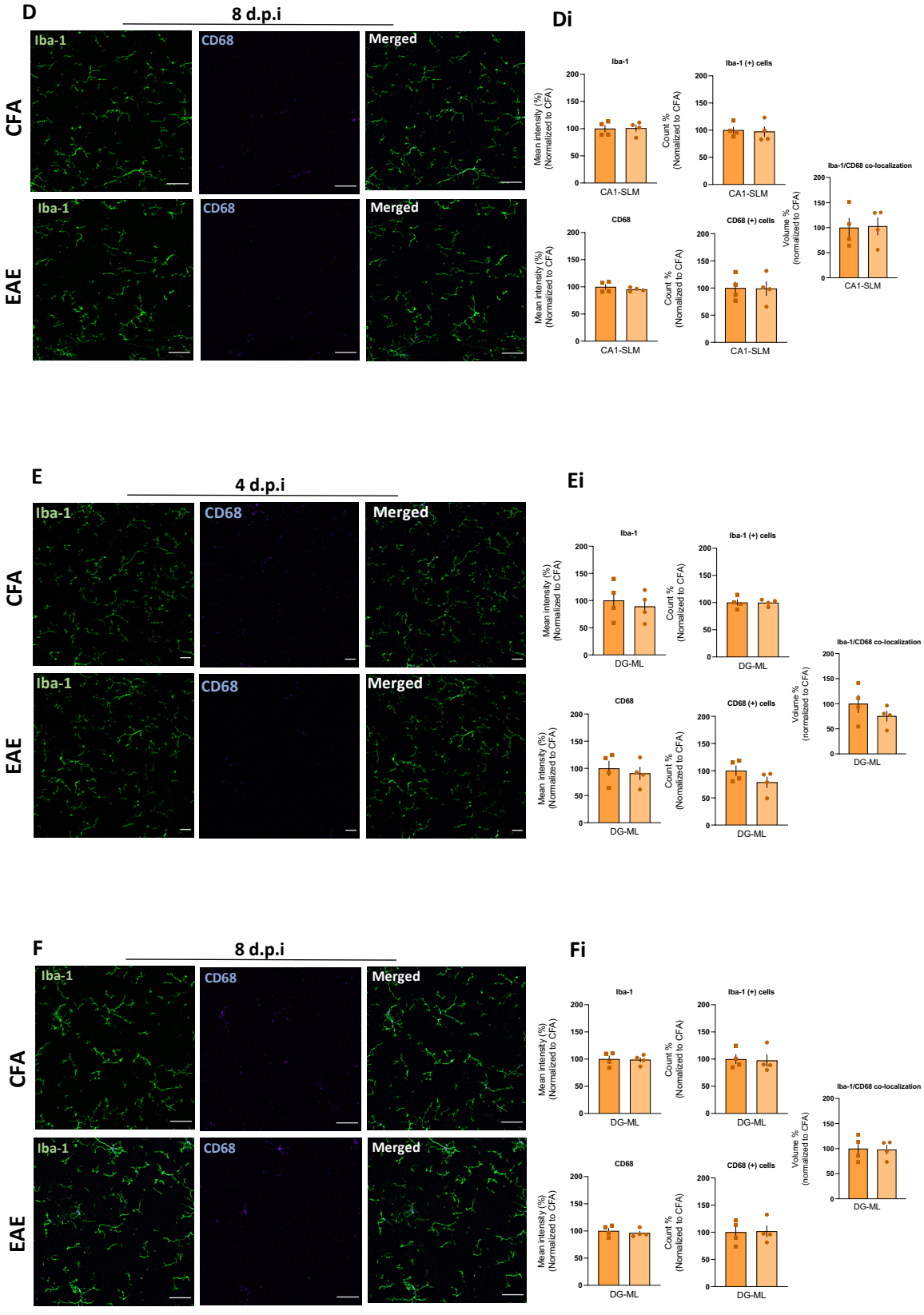


Figure 1.7 Representative figure of sagittal hippocampal section. White arrows indicate regions analyzed. From the top; CA1-str.R, CA1-SLM and DG-ML. Scale bars, 200 μm.


A
4 d.p.i
Iba-1
CD68
Merged
CFA
EAE
Ai
Iba-1
Iba-1 (+) cells
CD68
CD68 (+) cells
Iba-1/CD68 co-localization
Mean intensity (%) (Normalized to CFA)
Count % (Normalized to CFA)
Volume % (normalized to CFA)
CA1-Str.R
B
8 d.p.i
Bi
C
4 d.p.i
Ci
CA1-SLM


D
8 d.p.i
Iba-1
CD68
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CFA
EAE
Di
Iba-1
Iba-1 (+) cells
Iba-1/CD68 co-localization
CD68
CD68 (+) cells
Mean intensity (%) (Normalized to CFA)
Count % (Normalized to CFA)
Volume % (normalized to CFA)
CA1-SLM
E
4 d.p.i
Ei
DG-ML
F
8 d.p.i
Fi

Figure 1.8 Iba-1 and CD68 expression levels are unaltered in the asymptomatic EAE hippocampus mice. A, B, C, D, E ,F) Representative maximum intensity plane of Iba-1 (left), CD68 (middle) and Iba-1/CD68 co-localization (right). **Ai, Bi, Di, Ei, Fi)** Quantification of Iba-1 mean fluorescence intensity and positive cells number (top), CD68 mean fluorescence intensity and positive cells number (down) and Iba-1/CD68 co-localized volume (right), in the **A, Ai, B, Bi)** CA1-str.R at **A, Ai)** 4.p.i and at **B, Bi)** 8 d.p.i; in the **C, Ci, D, Di)** CA1-SLM at **C, Ci)** 4.p.i and at **D, Di)** 8 d.p.i. and in the **E, Ei, F, Fi)** DG-ML at **E, Ei)** 4.p.i and at **F, Fi)** 8 d.p.i, in the CFA (up) and EAE (down) mice. All values are: normalized to CFA control mice. EAE:CFA, N=4. Scale bars, 20 μm. All data for this and subsequent immunohistochemical quantification graphs are presented as mean ± SEM. Unless stated otherwise, a two-tailed unpaired t-test was used, significance at $p < 0.05$.

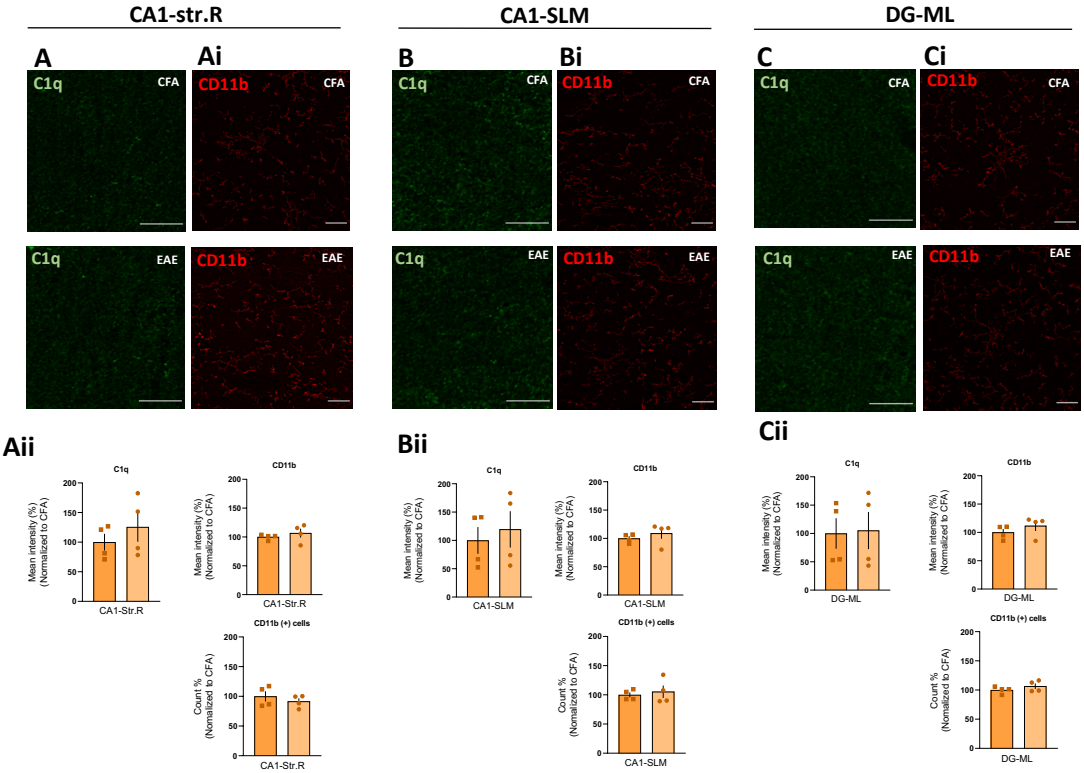
4.2. Classical complement pathway activation in the asymptomatic stage of EAE

To determine if the EAE hippocampus also exhibits higher expression levels of the classical complement cascade which could be a sign for vulnerable synapses, we analyzed the immunoreactivity of the initiating classical pathway protein, C1q (Mortensen et al., 2017) and CD11b, which pairs with CD18 to form the complement receptor CR3 target for C3 protein heterodimer (Wagner et al., 2001).

4.2.1. C1q and CD11b protein levels are unaffected in the hippocampus of EAE mice at 4 d.p.i and 8 d.p.i

At days 4 and 8 after immunization, we found there was no significant difference in C1q immunofluorescence intensity in the CA1-str.R (4 d.p.i Figure 1.9 A and Aii, p=0.4; 8 d.p.i, Figure 1.9 D and Dii, p=0.56), CA1-SLM (4 d.p.i Figure 1.9 B and Bii, p=0.64; 8 d.p.i, Figure 1.9 E and Eii, p=0.72) or in the DG-ML (4 d.p.i Figure 1.9 C and Cii, p=0.9; 8 d.p.i, Figure

1.9 F and Fii, p=0.82), between EAE WT and CFA controls. Further, CD11b expression levels and immunolabelled positive cells number were not significantly different in EAE mice compared to CFA in either 4 or 8 d.p.i, in the CA1-str.R (4 d.p.i, Figure 1.9 Ai and Aii; p=0.45, p=0.4, for mean intensity fluorescence and number of positive cells respectively; 8 d.p.i, Figure 1.9 Di and Dii; p=0.06, p=0.8, respectively), CA1-SLM (4 d.p.i, Figure 1.9 Bi and Bii; p=0.156, p=0.3, respectively; 8 d.p.i, Figure 1.9 Ei and Eii; p=0.1, p=0.95 respectively) or the DG-ML (4 d.p.i, Figure 1.9 Ci and Cii; p=0.944, p=0.1, respectively; 8 d.p.i, Figure 1.9 Fi and Fii; p=0.1, p=0.6, respectively). CD11b is also used mainly as a marker for microglia in the brain (Jeong et al., 2013).



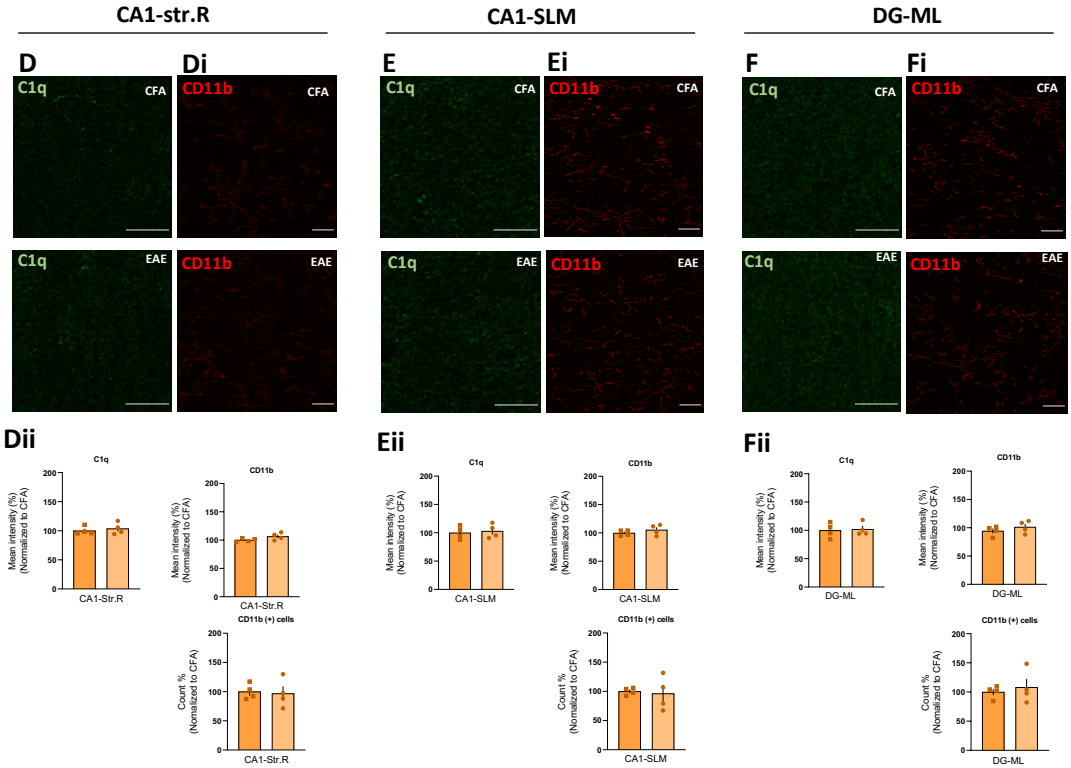


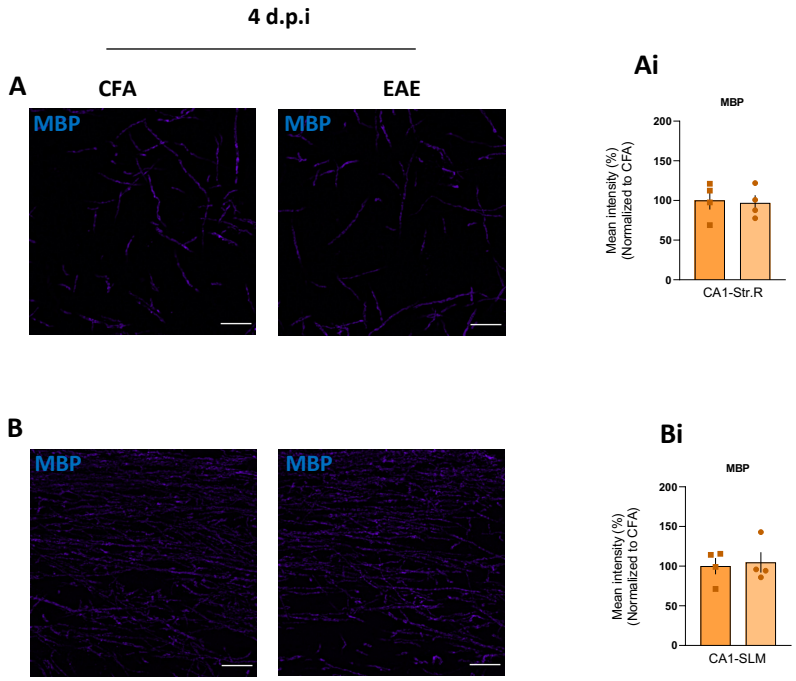
Figure 1.9 C1q and CD11b protein levels are unaffected in the hippocampus of EAE mice in the asymptomatic stage of the disease. Representative maximum intensity plane of C1q at 4 d.p.i in the **A)** CA1-str.R , **B)** CA1-SLM, and **C)** DG-ML and at 8 d.p.i in the same regions **D),E)** and **F)**, respectively. Representative maximum intensity plane of CD11b at 4 d.p.i in the **Ai)** CA1-str.R , **Bi)** CA1-SLM, and **Ci)** DG-ML and at 8 d.p.i in the same region **Di)**, **Ei)** and **Fi)**, respectively. in the CFA (up) and EAE (down) mice. For all images, values are normalized to CFA control mice; EAE:CFA, N=4. Scales bars for C1q figures= 10 μm and for CD11b figures= 20 μm). **Aii, Bii, Cii)** Quantification of C1q (left) and CD1bb mean fluorescence intensity (Right), and CD11b positive cells number (down) at 4 d.p.i and **Dii, Eii and Fii)** at 8 d.p.i.

4.3. Analysis of myelin basic protein (MBP) expression levels show no myelin disruption in the asymptomatic stage of EAE

The damage in the myelin sheath is the major pathological feature of MS (Popescu et al., 2013) and EAE (Band, 2015). Demyelination may lead to the axonal damage observed in the white and grey matter of MS patients

(Haines et al, 2011). Furthermore, demyelination contributes to hippocampal pathology in MS patients who suffered from cognitive deficits (Geurtset al., 2007).

We performed immunohistochemical staining for myelin basic protein (MBP), responsible for the myelin sheath organization (Boggs, 2006), in hippocampal sections from EAE at 4 and 8 d.p.i compared to CFA mice. We found no difference in MBP mean fluorescence intensity in the CA1-str.R (4 d.p.i, Figure 2 A and Ai; p=0.84; 8 d.p.i, Figure 2 D and Di; p=0.58), CA1-SLM (4 d.p.i, Figure 2 Bi and Bi; p=0.87; 8 d.p.i, Figure 2 E and Ei; p=0. 58)or DG-ML between the two groups (4 d.p.i, Figure 2 C and Ci; p=0.59; 8 d.p.i, Figure 2 F and Fi; p=0.44). These results indicate that in the absence of evidences autoreactive T cells (Acharjee et al., 2013) and in accordance with unaltered microglia activity, during the asymptomatic stages of the disease, hippocampal EAE mice do not exhibit any demyelinating phenotype.



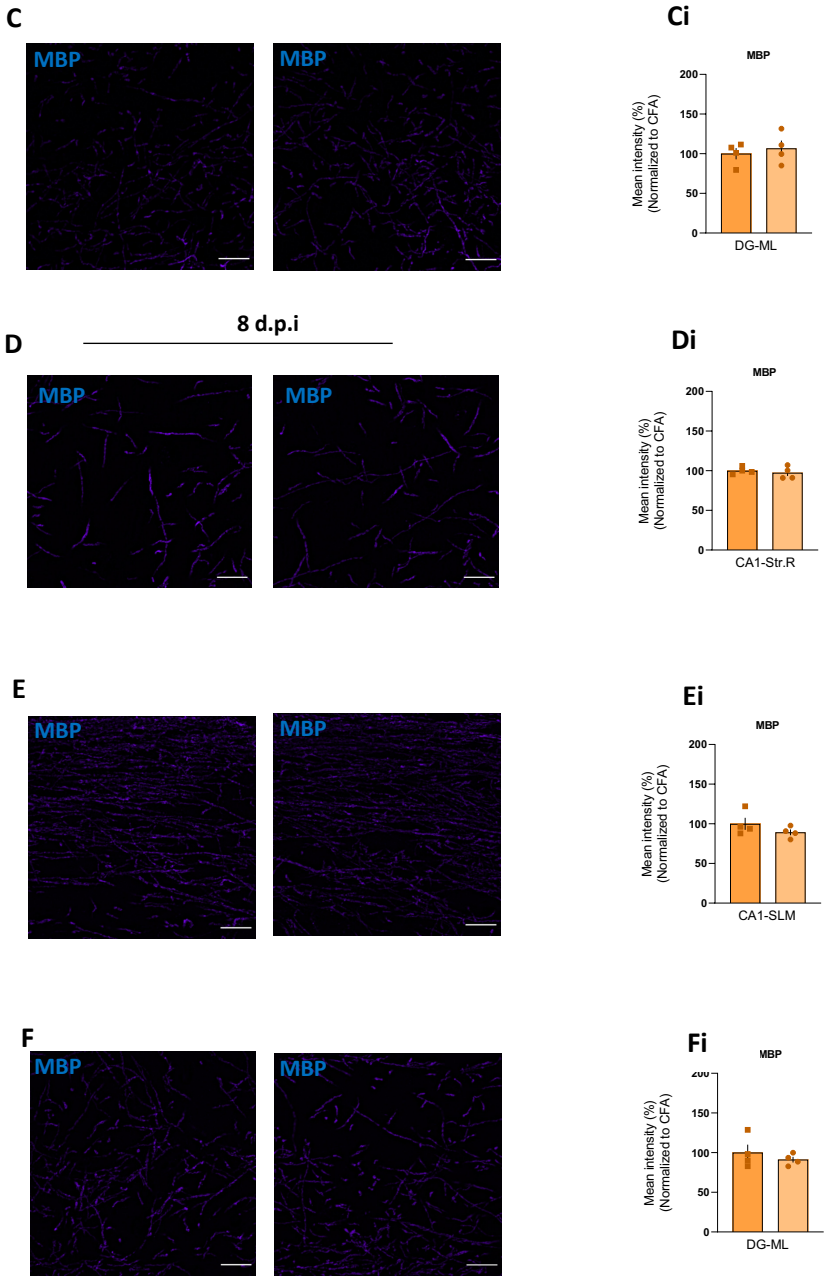


Figure 2 myelination levels are unchanged in the hippocampus of asymptomatic EAE mice. Maximum intensity projection images of MBP expression in the CA1-str at **A)** 4d.p.i and at **D)** 8 d.p.i, in the CA1-SLM at **B)** 4 d.p.i and **E)** 8 d.p.i and in the DG-ML at **C)** 4 d.p.i and **F)** 8 d.p.i, in the CFA (left) and EAE (right). Quantification of MBP mean fluorescence intensity normalized to CFA values, at 4 and d.p.i in the **Ai, Di)** CA1-str.R; **Bi, Ei)** CA1-SLM and **Ci, Fi)** DG-ML, respectively. Scale bars, 20 μm. EAE:CFA, N=4.

4.4. Characterization of hippocampal GABAergic and glutamatergic synapses in the asymptomatic stage of EAE

4.4.1.Hippocampal layer-specific increase in inhibitory presynaptic Vgat but not the postsynaptic Gephyrin

We also evaluated whether there was an increase or loss in the inhibitory synapses during the asymptomatic phase of EAE. Therefore, we used IHC to stain for the inhibitory presynaptic bouton marker vesicular GABA transporter (VGAT) and for the postsynaptic assembly protein (Gephyrin) along the CA1-str.R, CA1-SLM and DG-ML. We found there was no significant alteration in GABAergic postsynaptic density across the three hippocampal layers at the early time points after induction; 4 d.p.i (CA1-str.R Figure 2.1 Ai and Aii, p=0.14; CA1-SLM, Figure 2.1 Bi and Bii, p=0.32; DG-ML, Figure 2.1 Ci and Cii, p=0.11) and 8 d.p.i (CA1-str.R Figure 2.1 Di and Dii, p=0.7; CA1-SLM, Figure 2.1 Ei and Eii, p=0.41; DG-ML, Figure 2.1 Fi and Fii, p=0.78), between the EAE and the sham groups. Similar to Ghephyrin expression levels, at day 4, Vgat immunoreactivity was unaffected by EAE induction in the hippocampus analyzed regions (CA1-str.R Figure 2.1 A and Aii, p=0.86; CA1-SLM, Figure 2.1 B and Bii, p=0.6; DG-ML, Figure 2.1 C and Cii, p=0.45). However, 8 days after immunization, Vgat levels showed a significant increase in the EAE mice compared to CFA group, specifically in the CA1-str.R (Figure 2.1 D and Dii; p=0.0125) but not in CA1-SLM (Figure 2.1 E and Eii, p= 0.71) or in the DG-ML (Figure 2.1 F and Fii, p=0.72).

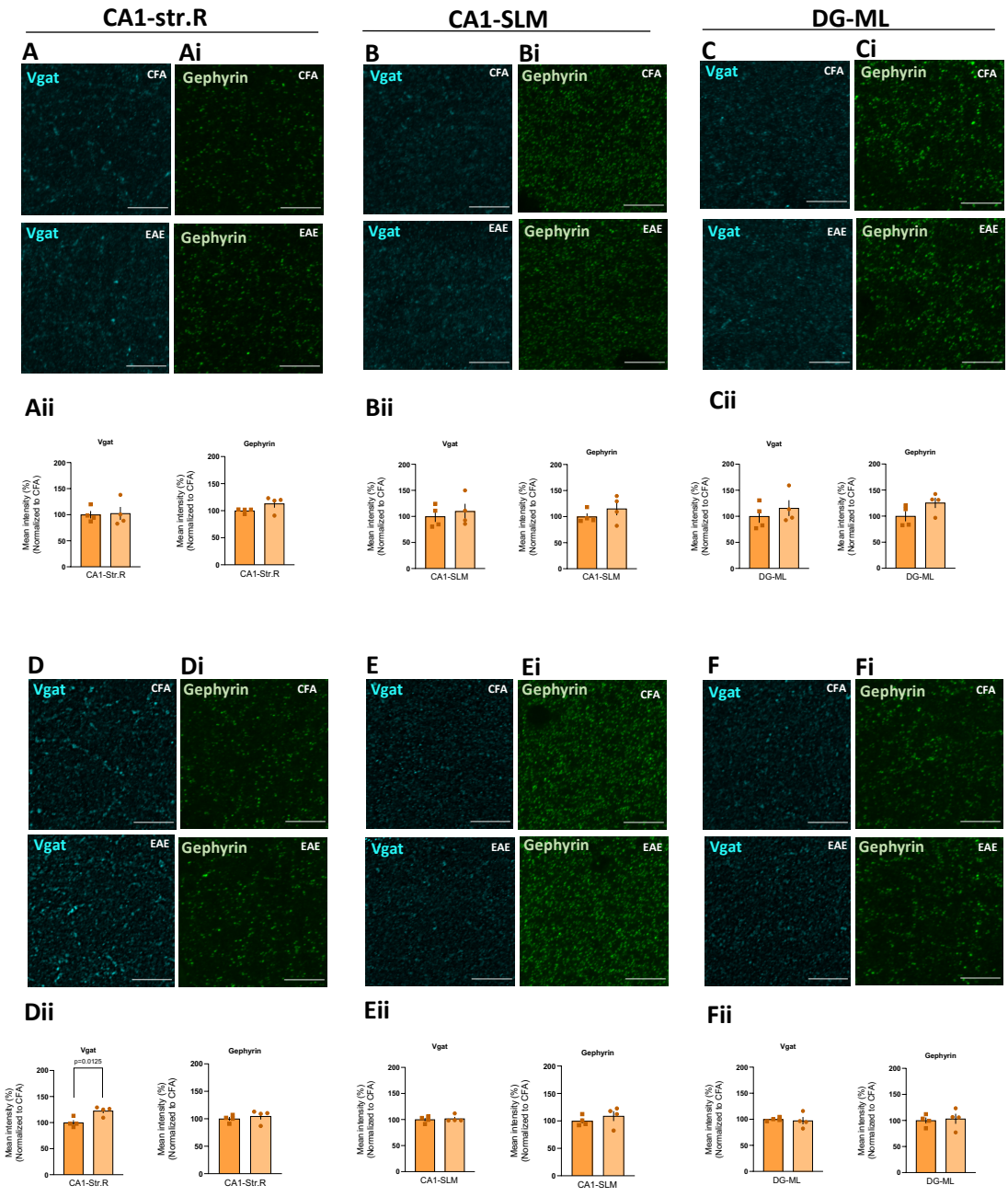


Figure 2.1 region-specific increase of Vgat expression in the hippocampus and no change in Gephyrin protein levels, during the asymptomatic stage of EAE.

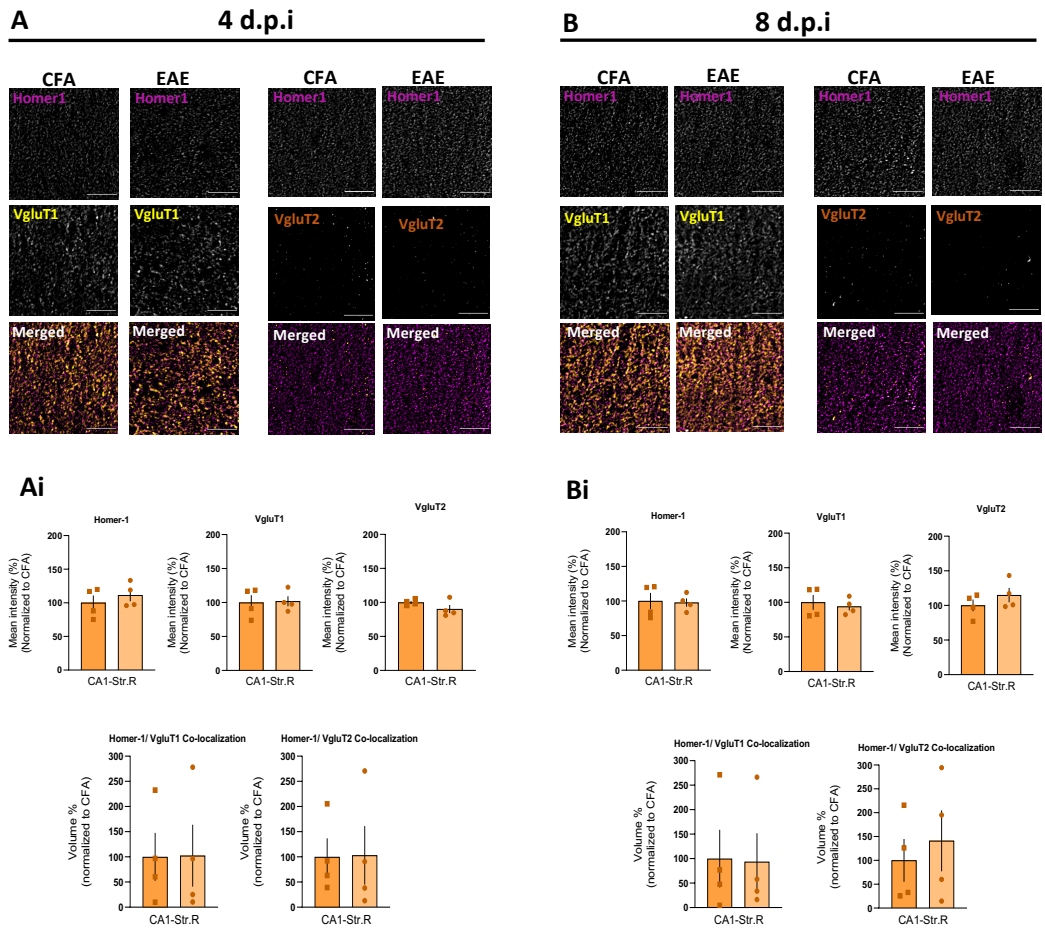
Representative maximum intensity plane of Vgat at 4 d.p.i in the **A)** CA1-str.R , **B)** CA1-SLM, and **C)** DG-ML and at 8 d.p.i in the same regions **D),E)** and **F)**, respectively. Representative maximum intensity plane of Gephyrin at 4 d.p.i in the **Ai)** CA1-str.R , **Bi)** CA1-SLM, and **Ci)** DG-ML and at 8 d.p.i in the same regions **Di)**, **Ei)** and **Fi)**, respectively. CFA (up) and EAE (down) mice. **Aii, Bii, Cii)** Quantification of Vgat (left) and Gephyrin mean fluorescence intensity (Right) at 4 d.p.i and **Dii, Eii and Fii)** at 8

d.p.i. For all images, values are normalized to CFA control mice; EAE:CFA, N=4. Scales bars = 10 μm.

4.4.2. Excitatory synapses are unaffected in the asymptomatic EAE mice

Glutamate is the major excitatory neurotransmitter in the CNS, and its extracellular concentration is tightly regulated by multiple mechanisms to avoid excitotoxicity phenomena, which is often associated with neuronal damage and neurodegenerative mechanism (Centonze et al., 2010). Increase in the glutamatergic transmission has been widely associated with the synaptic pathology of the hippocampus and with the cognitive deterioration in MS patients and animal models (Mandolesi et al., 2015). Thus, we investigated vesicular glutamate transporter VgluT1+ ,VgluT2+ and the scaffold protein Homer-1+ synapses across the CA1 and DG to determine whether all types of excitatory pre-synapses and post-synapses are affected by EAE induction before motor symptoms appear. At 4 and 8 d.p.i, we found no significant difference in the immunofluorescence intensity signal of VgluT1, VgluT2 and Homer-1 within the CA1-str.R (Homer-1: 4 d.p.i, Figure 2.2 A and Ai, p=0.44 ; 8 d.p.i, Figure 2.2 B and Bi , p= 0.87. VgluT1: 4 d.p.i, Figure 2.2 A and Ai, p= 0.89; 8 d.p.i, Figure 2.2 B and Bi, p= 0.64. VgluT2: 4 d.pi, Figure 2.2 A and Ai, p= 0.17; 8 d.p.i, Figure 2.2 B and Bi, p= 0.30) , CA1-SLM (Homer-1: 4 d.pi, Figure 2.2 C and Ci, p= 0.41; 8 d.p.i, Figure 2.2 D and Di, p= 0.61. VgluT1: 4 d.pi, Figure 2.2 C and Ci, p= 0.94; 8 d.p.i, Figure 2.2 D and Di, p= 0.87. VgluT2: 4 d.p.i, Figure 2.2 C and Ci, p= 0.99; 8 d.p.i, Figure 2.2 C and Ci, p= 0.95) and DG-ML (Homer-1: 4 d.pi, Figure 2.2 E and Ei, p= 0.84; 8 d.p.i, Figure 2.2 F and Fi, p= 0.97. VgluT1: 4 d.pi, Figure 2.2 E and Ei, p= 0.84; 8 d.p.i, Figure 2.2 F

and Fi, p= 0.68. VgluT2: 4 d.pi, Figure 2.2 E and Ei, p= 0.68; 8; 8 d.p.i, Figure 2.2 F and Fi, p= 0.9), between EAE and CFA mice. Further, we tested if there was an increase or decrease in the spatial overlap between pre- and post- excitatory synapses terminals, therefore we exanimated the co-localization between synapses markers mentioned above, and there was no significant change in VgluT1 and Homer1 or in the VgluT2 and Homer1 co-localized volumes within the three investigated hippocampal regions at 4 d.p.i (Figure 2.2 A, Ai, C, Ci, E, Ei) or at 8 d.p.i (Figure 2.2 B, Bi, D, Di, F, Fi) of EAE mice compared to CFA controls. Collectively, these data indicate that the number of excitatory synapses in the SC and PP are unaltered in the asymptomatic stage of EAE.



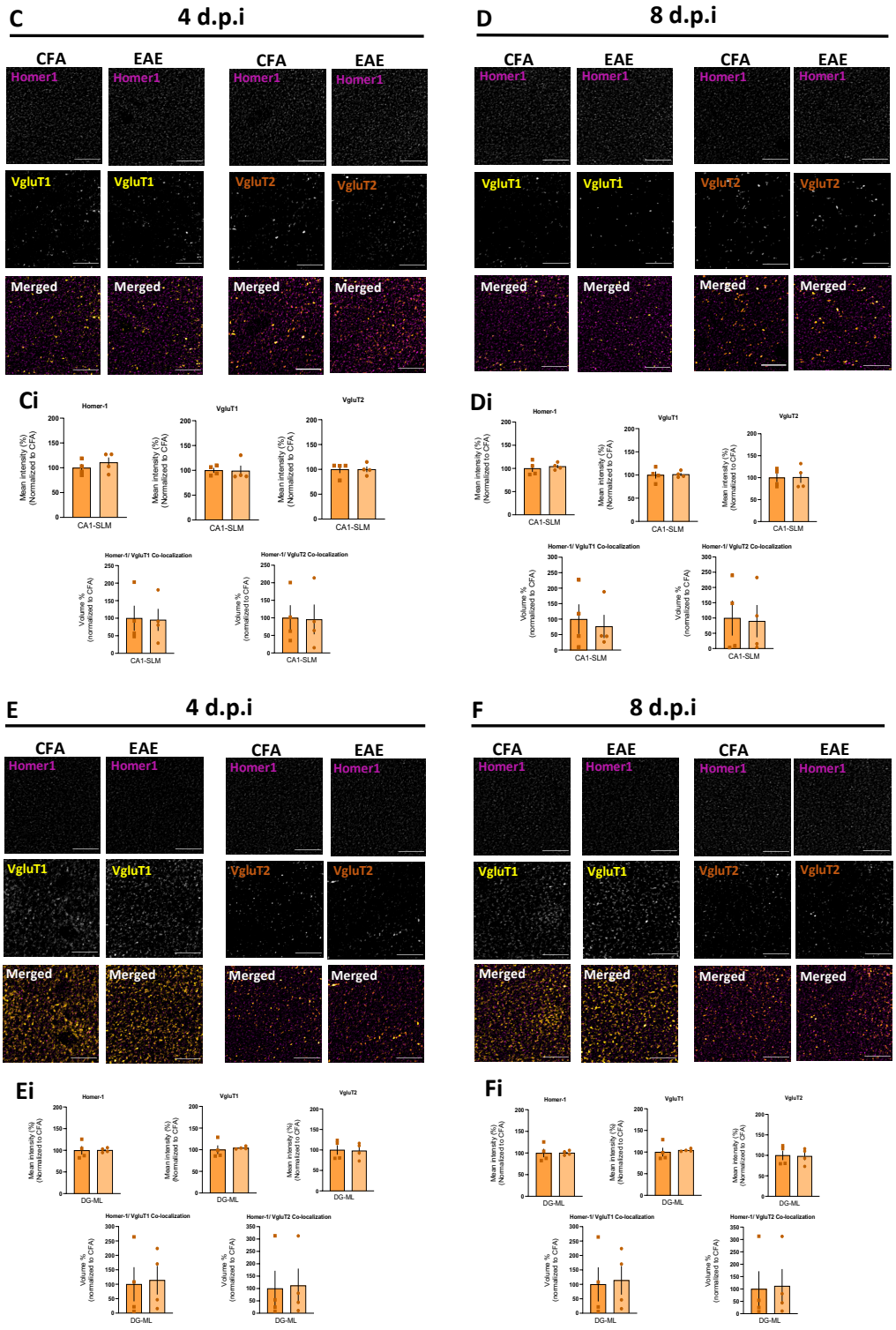


Figure 2.2 Glutamatergic synapses are unaffected in the EAE mice in the asymptomatic stage. Representative maximum intensity images of Homer-1 (up), VgluT1 (middle), VgluT2 (middle) and Homer-1/ VgluT1 or Homer-1/VgluT2 co-localization (down) in the EAE mice (right) and CFA controls (Left) in the **A)** CA1-str. R area in days 4 (left) and 8 (right) after immunization, **B)** CA1-SLM region in days 4 (left) and 8 (right) after

immunization and in the **C)** DG-ML at 4 (left) and 8 d.p.i (right). Quantification of Homer-1 (left) and VgluT1 (middle) and VgluT2 (right) and theri co-localized volume (down) at days **Ai, Ci and Ei**) 4 and **Bi, Di and Fi)** 8 post-immunization. For all images, values are normalized to CFA control mice; EAE:CFA, N=4. Scales bars = 20 µm.

4.5. Synaptic transmission and plasticity in the hippocampal CA1 and DG regions in the asymptomatic stage of EAE

Next, we sought to determine whether in the hippocampus SC and PP are present functional alterations during the asymptomatic EAE clinical course. Therefore, we investigated three electrophysiological proprieties of the hippocampus in CA3-CA1 Schaffer collateral pathway and in the DG-ML MPP (Figure 2.3); we analyzed the basal synaptic transmission (BS) by measuring the I/O responses, the short term plasticity referred as PPF or PPD, most often used to understand if the observed response is due to a pre-synaptic or post-synaptic effects; this STP is evaluated by the paired pulse ration (PPR) as a measure of the probability of transmitter release at the synapse, and the NMDA-dependent LTP by measuring changes in fEPSP slope with time after a theta burst stimulation (TBS) in the SC pathway and high- frequency stimulation (HFS) in the MPP.

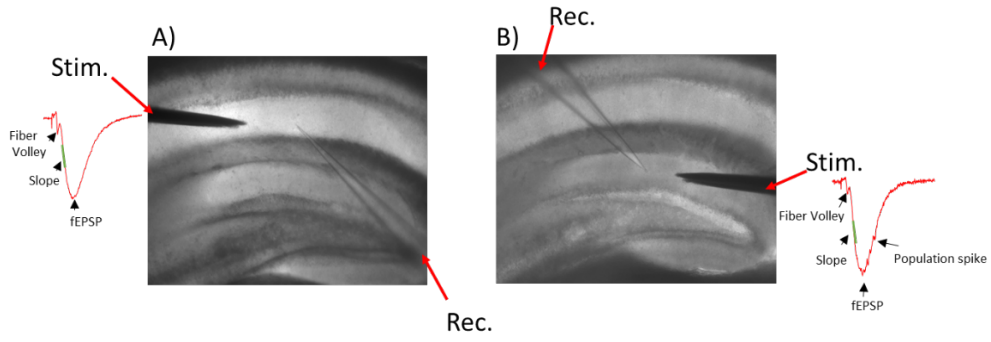


Figure 2.3 Schematic representation of the acute hippocampal slice configuration for typical fEPSP response in the A) CA1-SC pathway and B) DG-ML MPP.

4.5.1. Basal synaptic transmission, short term plasticity and Long term potentiation in the CA1 of asymptomatic EAE mice

To investigate the hippocampus synaptic functional properties and plasticity in the CA3-CA1 SC pathway of the hippocampus in the asymptomatic EAE mice. First, we characterized basal synaptic transmission by analyzing the I/O curves which is an electrophysiological index used to assess the strength of neural response to the increasing stimuli (Carvalho et al., 2009), we found no changes between EAE and CFA mice, at 4 d.p.i (Figure 2.4 A, Ai) and 8 d.p.i (Figure 2.4 B, Bi), in the fEPSP slope , as well as the fiber volley, values in function of stimulus intensity. Then, we measured the changes in transmitter release probability. PPR values in the CA1 of EAE and CFA mice was similar in both 4 and 8 d.p.i (Figure 2.5 A and B , respectively). Next, we evaluated the LTP induction levels on CA1 pyramidal cells acute hippocampal slices obtained from EAE and CFA mice and the observed potentiation was not significantly changed between both groups at day 4 after immunization (Figure 2.6 A). However, in the CA1 there is a trend of increase in the fEPSP slope in the EAE mice albeit not significant at 8 d.p.i (Figure 2.6 B) which is not attributed to an increase in the number of recruited afferents but to the response of the stimulated afferent at the baseline intensity since there no increase in the recorded fiber volley at the same synapses in function of increased stimulation.

Based on the data discussed above, BS, STP and LTP in SC CA1 synapses were unaltered in both early time points before the onset of motor symptoms. However, the trend of increased LTP observed in day 8 after immunization should be more investigated by increasing the N of animals to statistically confirm or deny the data.

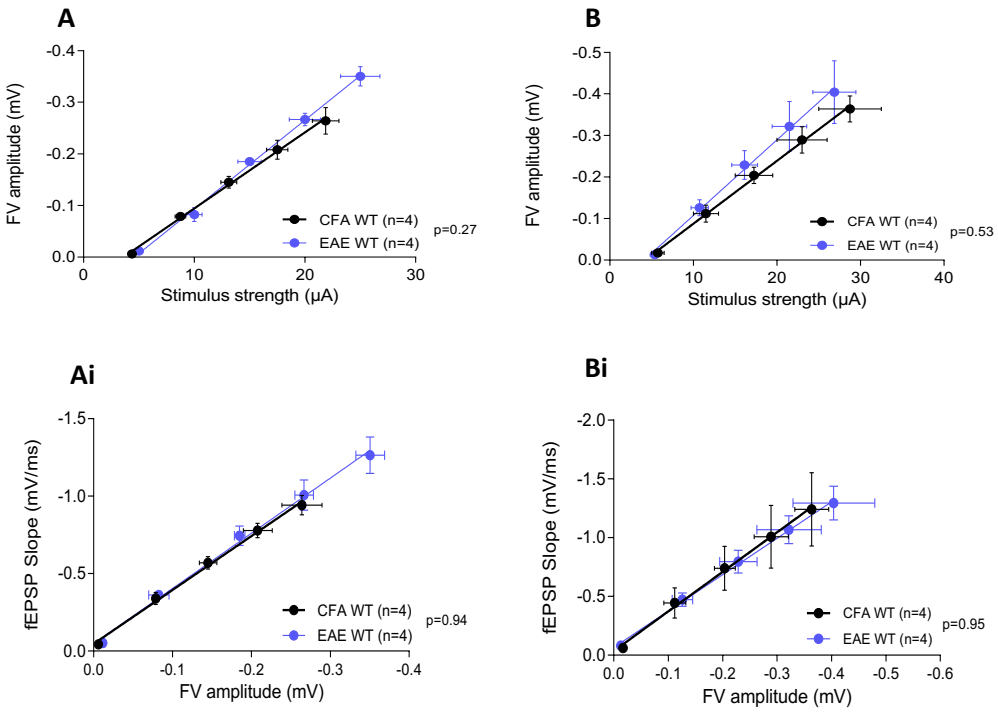


Figure 2.4 Input-output relationship at the CA1. Fiber volley magnitude plotted against stimulus intensity increments at **A)** 4 d.p.i and **B)** 8 d.p.i, and Fiber volley of evoked fEPSPs plotted against stimulus intensity increments at **Ai)** 4 d.p.i and **Bi)** 8 d.p.i, in EAE mice (Blue) and CFA controls (Black). EAE:CFA, N=4.

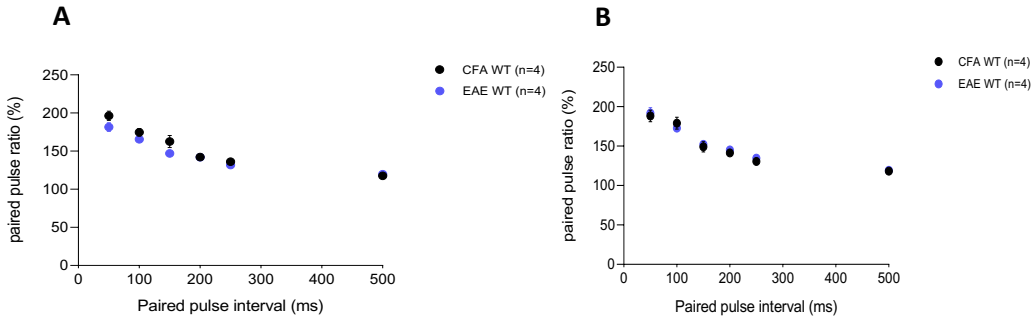


Figure 2.5 Paired-pulse facilitation at the CA1. Ratio of second applied stimulus fEPSP slope to the first stimulus fEPSP slope plotted against interstimulus delay (50, 100, 150, 200, 250, 500 ms) at **A)** 4 d.p.i and at **B)** 8.d.p.i. EAE:CFA, N=4.

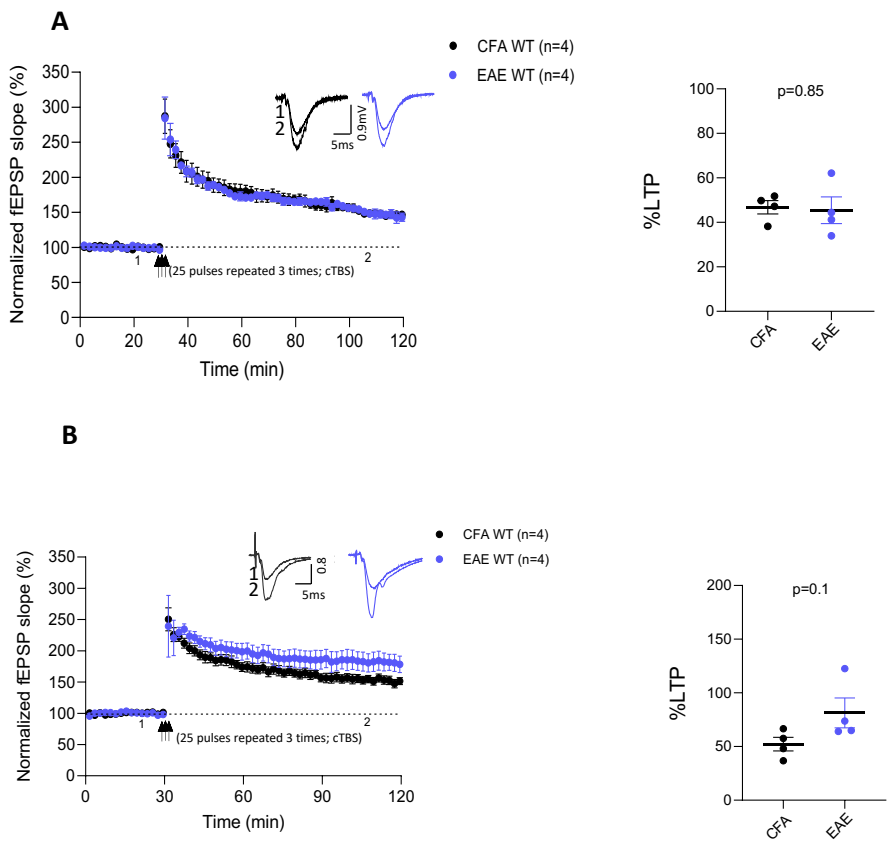


Figure 2.6 LTP at the CA1. All panels represent experimental data obtained from n=4 slices obtained from 4 animals. cTBS was delivered at T=30 min after stable baseline. Recorded in EAE (blue) and CFA (black) at **A)** 4 d.p.i and **B)** 8 d.p.i.

4.5.2. Basal synaptic transmission, short term plasticity and Long term potentiation in the DG of asymptomatic EAE mice

Then, we performed same electrophysiological recordings mentioned above on the DG-ML MPP. Unlike the CA1, DG is characterized by higher inhibitory tone which negatively impacts the LTP induction (Sawada et al., 1994), therefore, we perfused 10 μM of bicuculine in order to

characterize the synaptic plasticity and neuronal transmission in this region of the hippocampus.

In order to determine if there was any alteration of the basal synaptic transmission, STP and LTP expression in the DG-ML MPP, we analyzed the I/O curves made by increasing the stimulus value, we found that there was no significant change in the fEPSP slope for every response against stimulus intensity in the 4 (Figure 2.7 A, Ai) and 8 d.p.i (Figure B, Bi) in the EAE mice compared to CFA. The PPR, as well, was not changed between both groups in the two early time points (4 d.p.i; Figure 2.8 A, and 8 d.p.i; Figure2.8 B).

Following induction LTP with HFS, we observed no significant change at 4 (Figure 2.9 A) or at 8 d.p.i (Figure 2.9 B) between EAE and sham group.

Taken together, electrophysiological data are consistent with IHC staining results i.e. the hippocampus synaptic integrity, and the non apparent demyelination or microglia-complement over-activation ensures that there was no alteration in the hippocampal synaptic transmission and plasticity.

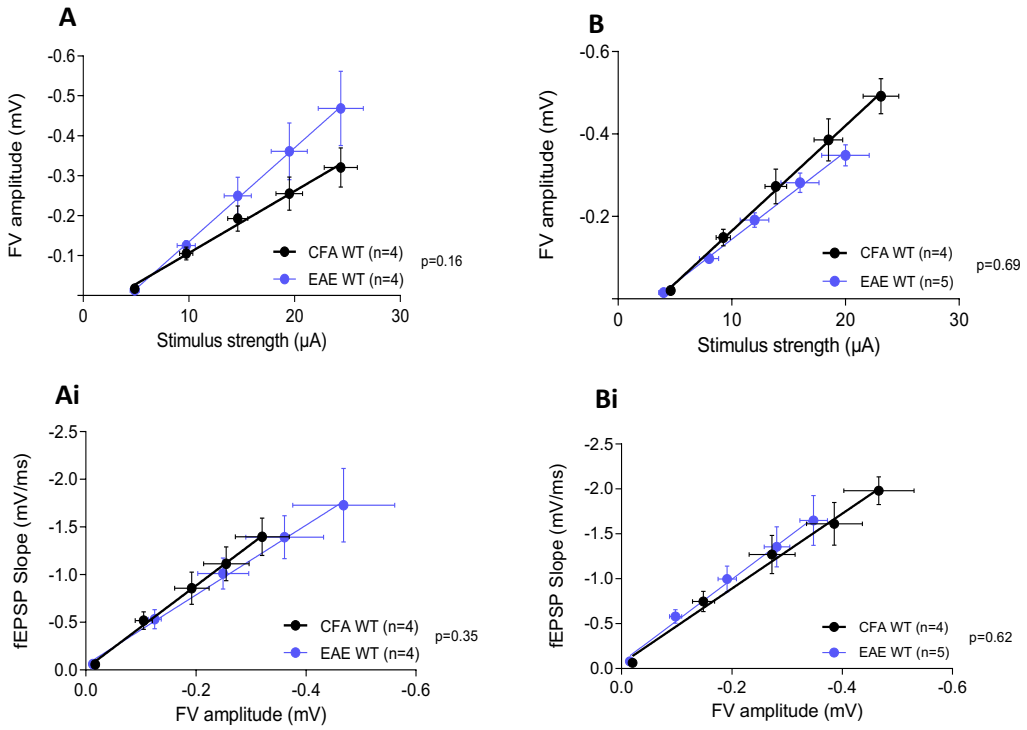


Figure 2.7 Input-output relationship at the DG. Fiber volley magnitude plotted against stimulus intensity increments at **A)** 4 d.p.i and **B)** 8 d.p.i, and Fiber volley of evoked fEPSPs plotted against stimulus intensity increments at **Ai)** 4 d.p.i and **Bi)** 8 d.p.i, in EAE mice (Blue) and CFA controls (Black). EAE: CFA, N=4 at 4 dp.i. EAE, N=5; CFA, N=4 at 8 d.p.i.

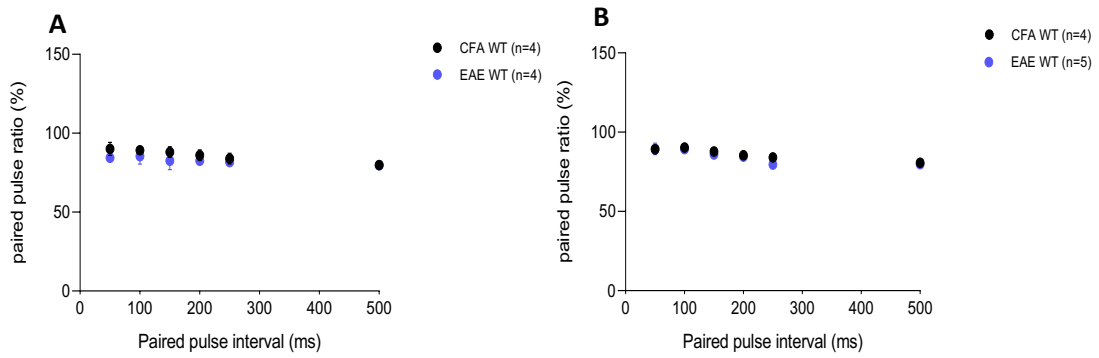


Figure 2.8 Paired-pulse facilitation at the DG. Ratio of second applied stimulus fEPSP slope to the first stimulus fEPSP slope plotted against interstimulus delay (50, 100, 150, 200, 250, 500 ms) at **A)** 4 d.p.i and at **B)** 8.d.p.i. EAE: CFA, N=4 at 4 dp.i. EAE, N=5; CFA, N=4 at 8 d.p.i.

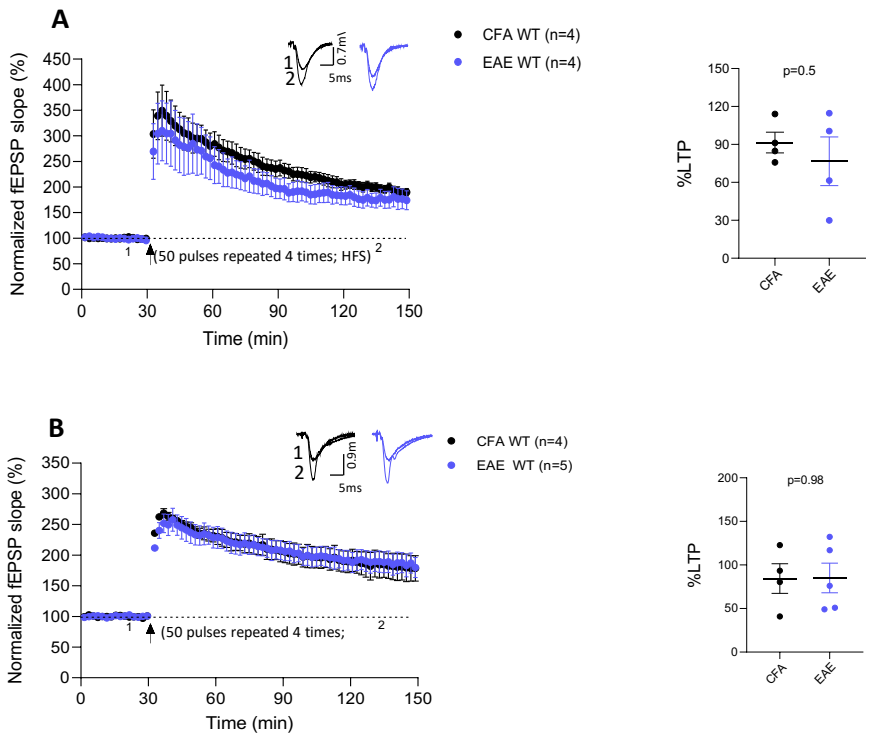


Figure 2.9 LTP at the DG. A) LTP experimental data obtained from EAE slices (Blue) and CFA (black) at **A)** 4 d.p.i and **B)** 8 d.p.i. EAE; n=5 slices from 5 animals. CFA; n=4 slices from 4 animals) at 8 d.p.i. Tetanus stimulation was delivered at T=30 min after baseline.

4.6. C3,CD11b and Trem2 are required for the EAE full development but not for the disease onset

Next, we questioned how the C3 complement protein and microglia phagocytic receptors C3R and Trem2 impact the EAE clinical progression. Therefore, we immunized WT, C3 KO, CD11b KO and Trem2 KO mice with MOG35-55 emulsion to determine their involvement in driving the disease pathology. The effect of genetic deletion of the classical complement components C3, CD11b and C1q has been deeply

investigated in several previous studies. As reported, C3 and CD11b loss of function protected against the EAE by inducing in immunized mice less motor impairment and significant delay in the disease onset (Nataf et al., 2000; Bullard et al., 2005; 2007; Szalai et al., 2007; Hammond et al., 2020), but not in the C1q KO mice in which C1q genetic deletion did not alter the EAE disease course (Hammond et al., 2020). Moreover, CPZ induced model of MS in Trem2 KO mice has shown an exacerbation in the clinical motor manifestations and demyelination progress (Cantoni et al., 2015).

Compared to their WT littermates, C3 KO resulted in less severe EAE motor deficits with a significant decrease in the mean clinical score both in the recovery stage (20 to 22 d.p.i) and during the chronic phase (28 to 30 d.p.i). Nevertheless, C3 deletion had no effect on the disease onset and on the peak clinical score (12 to 18 d.p.i) (Figure 3 A, Ai). Furthermore, inducing EAE in CD11b KO mice have shown almost same disease progression pattern as C3 KO mice, demonstrating an attenuated EAE when compared to WT mice; moreover, the disease peak and the clinical score in the chronic phase were significantly lower than the EAE WT mice (Figure 3 B, Bi). However, for EAE CD11b KO, more animals are needed to confirm this data.

Unexpectedly, deletion of Trem2 leads to markedly reduced clinical motor impairments score compared to their WT littermates (Figure 3 C, Ci) and more resistance to the EAE deleterious effects if compared to C3 and CD11b KO mice. We found that Trem2 KO significantly reduced the peak of the disease, improve recovering and significantly reduces the chronic progression of EAE . However, similar to C3 KO and CD11b KO mice, Trem2 genetic deletion had no effect on the onset of the disease, similar to

their WT littermates, they developed the first motor symptoms between days 10 and 11 after immunization.

This result is consistent with previous publications about the important role played by complement system pathways in EAE physical impairments due to the degeneration of the white matter. However, these data open a new insight toward the real role of Trem2 in the EAE inflammatory response.

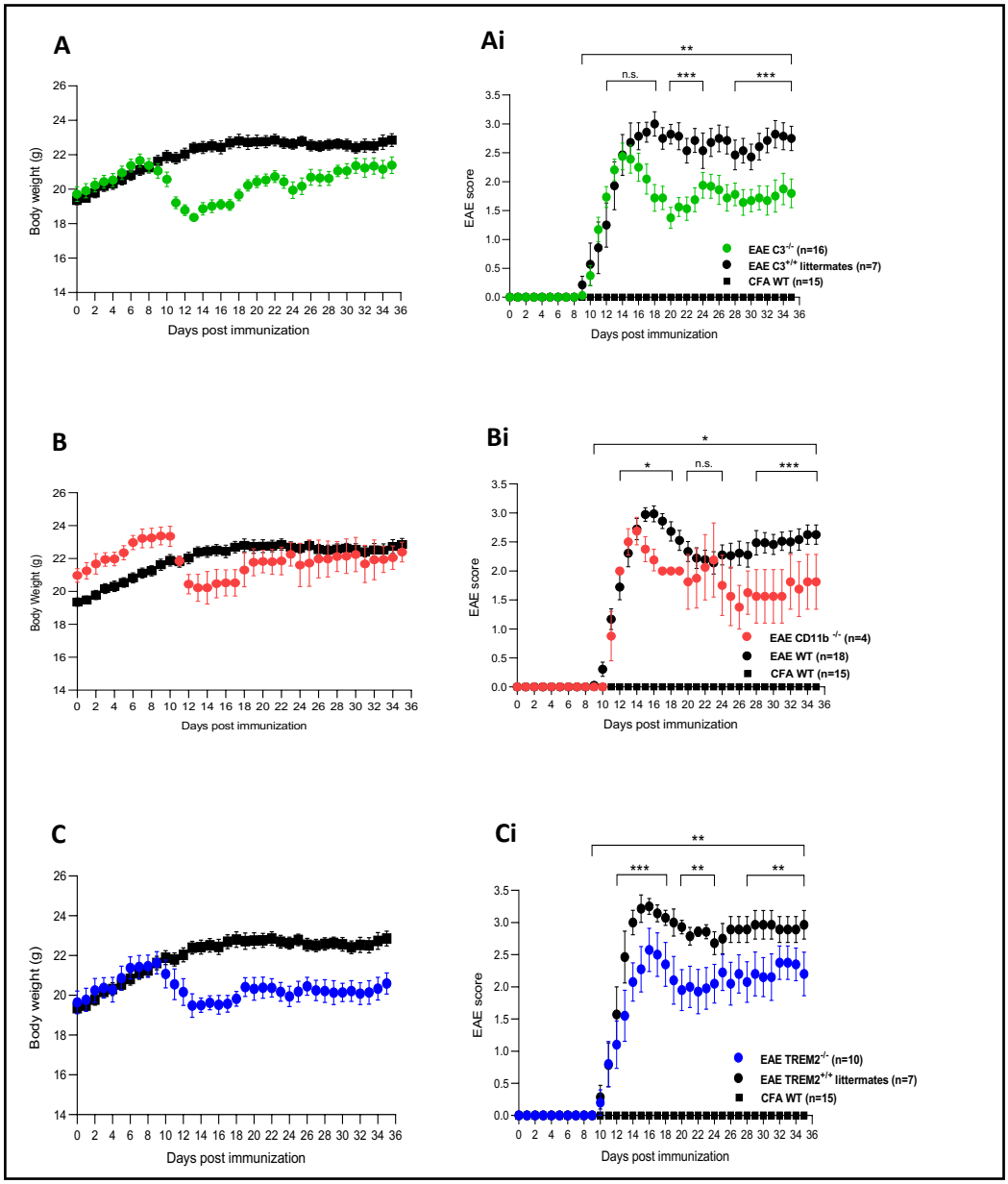


Figure 3 Clinical MOG 33-55 induced EAE score is attenuated in C3, CD11b and Trem2 KO mice. EAE clinical score and bodyweight in **A, Ai)** $C3^{-/-}$ and their littermates, **B, Bi)** $Cd11b^{-/-}$ compared to WT non littermate mice, and **C, Ci)** $Trem2^{-/-}$and their littermates. CFA score is reported in all the EAE score graphs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

5. Discussion and future directions

Cognitive decline and memory impairment are common features of multiple sclerosis (Hulst et al., 2013), including hippocampus-dependent memory deficits (Mancini et al., 2017). These symptoms generally occur early in MS patients and before the manifestation of motor disability in animal models of MS (Kim et al., 2012; Acharjee et al., 2013; McNicholas et al., 2017). Nevertheless, the involvement of the hippocampus during the presymptomatic stage of the disease still needs to be fully understood. However, the contribution of microglia to MS pathology and its detrimental effects on the synaptic integrity of the hippocampus is recently emerging (Mancini et al., 2017). Dysfunctions of the complement system and of the microglial phagocytic receptor Trem2, are emerging as key a components in many neurodegenerative diseases including MS (Jay et al., 2017; Dalakas et al., 2020).

Therefore, in this thesis, we have focused on the effect of early MOG 35-55 induction pathological mechanism on the microglia-synapse interaction in the hippocampus of EAE, the most used animal model to study the immunopathogenesis of MS (Constantinescu et al., 2011). We have investigated the potential contribution of microglia and the classical complement pathway to hippocampal synaptic changes at the early stage of disease, in order to determine the first pathogenic mechanisms affecting this brain region.

Firstly, we performed immunohistochemistry to investigate the proliferation and activation of the microglial cells in the CA1 and DG areas of the hippocampus. We did not observe significant differences in the overall microglia number or on the expression level of the phagocytic marker CD68 in the EAE mice compared to their CFA controls, suggesting that microglia may potentially be involved later in hippocampus pathology depending on the disease progression. These results were partly expected since it was previously reported that in the hippocampus of presymptomatic EAE there was no change regarding microglia activity (Asharjee et al., 2013), proinflammatory cytokine release or Tcell infiltration (Yang et al., 2013; Acharjee et al., 2013).

The overall goal of this study was also to investigate the contribution of classical complement pathway to the synaptic pathology of the hippocampus. Thus, we started by analyzing the protein expression levels of the main classical complement components, C1q and C3R. To detect the immunoreactivity of C3R, we have used antibody against the subunit CD11b which is used also as a marker for microglia activation. Then again, we confirmed that in the CA1 and DG there was no increase in the microglia activity and we did not find any changes in C1q protein levels. The complement proteins including C1q and C3 have been predominantly found deposited on the active white and gray matter lesions (Michailidou et al., 2015; Tatomir et al., 2017), and there are no evidences about their presence in the presymptomatic phase of EAE. Moreover, C1q gene deletion did not protect form EAE severity and had minor effect on hippocampal synaptic damage at the chronic phase of EAE (Hammond et al., 2020). To sum up, during the presymptomatic stage of EAE, at days 4 and 8 after immunization, microglia did not show increased phagocytic

activity and C1q-CD11b expression levels were not different from the controls, suggesting that a mechanism similar to the developmental synaptic pruning is not present in the early phase of EAE.

As the demyelination is the one of the hippocampal pathological hallmark in MS (Rocca et al., 2018), we analyzed the immunostaining for the myelin essential component, MBP (myelin basic protein). There was no significant difference between EAE and CFA on MBP expression in the hippocampus at 4 and 8 d.p.i.. Demyelination is frequently associated with increased inflammation and synaptic density loss (Dutta and Trapp, 2011), which were not observed in EAE within the two chosen time points.

Microglia is not the only active player responsible for a potential hippocampal structural or functional alteration during MS and in EAE model, but also other cellular mechanisms such as the overactivation of the astrocytes and the activation of the hypothalamic–pituitary–adrenal axis (HPA axis), may contribute to the increase of inflammatory responses in the brain and induce synaptic damage in the hippocampus (Ponath et al., 2018; dos Santos et al., 2019). Therefore, we sought to identify any abnormality in the hippocampus at the presymptomatic stage of EAE. Thus, we analyzed the excitatory and the inhibitory synaptic density in the CA1 and DG by labelling for their pre-and post-synaptic markers. Glutamatergic synapses were not affected before the disease onset, as well as, the post-synaptic site of GABAergic neurons. Nevertheless, at day 8 after immunization, we observed a significant increase in the immunoreactivity of the GABAergic pre-synaptic vesicular transporter (Vgat), specifically in the CA1-str.R. It can be hypothesized that increased Vgat and consequent GABA release at CA1 synapses might represent an initial protective mechanism to attenuate the increase of

glutamate release and to prevent excitotoxicity (Muir et al., 1996) which is associated with the later stages of EAE (Mandolesi et al., 2016). On the other hand, enhanced Vgat expression might also represent a compensatory mechanism to counteract the reduced inhibitory transmission associated with EAE hippocampus (Nistico' et al., 2013). Therefore further experiments are needed to clarify the role of GABA and glutamate, as well as the Excitatory/Inhibitory balance, during the different stages of EAE. Indeed, increased glutamatergic transmission has been proposed as major determinant for synaptic pathology in the grey matter in MS and EAE (Centonze et al.,2010). Conversely, in the presymptomatic stage of EAE analyzed, we did not find any alteration in the glutamatergic pre- and post-synaptic terminals associated with the increased levels of Vgat. On the other hand, increased GABA neurotransmission has been proposed as a mechanism to impaired memory in AD (Wu et al., 2014). Whether the increased expression of GABA transporter is beneficial or detrimental in EAE model should be further investigated by electrophysiological experiments. Moreover, similarly to our results, increasing in GABA synaptic immunoreactivity was found in the CA1 of MS patients within the active and non-active lesions (Kiljan et al., 2019), further, in vivo electrophysiological recording in the CA1 region in EAE rats showed impaired synaptic transmission and inhibited LTP with a decrease in the PPR which was associated to an increase in the GABAergic inhibitory effects (Mosayebi et al., 2016). Therefore, to understand the effect of EAE induction on synaptic transmission and plasticity at the early stages, we analyzed the field excitatory postsynaptic potentials (fEPSPs) slope and LTP in the DG and CA1 regions. We did not find any significant changes in the CA1 or DG

electrophysiological responses in EAE mice compared to their controls at days 4 and 8 post-immunization.

To determine the role of microglia phagocytic activity on the EAE onset and progression, we focused on two mechanisms; C3-mediated opsonization and Trem2-mediated phagocytosis. Therefore, we induced EAE on Trem2, C3 and CD11b knock-out (KO) mice. We found that KO of C3 or in its receptor subunit CD11b, significantly attenuated EAE disease severity. C3 ,as well as CD11b, had no effect on the disease onset, indeed there was no difference when compared to immunized WT mice. The deleterious effects of C3 and its receptor C3R on EAE clinical progression have been widely investigated (Szalai et al., 2007; Smith et al., 2008; Bullard et al., 2007). Additionally, complement system demonstrated to be a good potential therapeutic target; inhibitors of complement pathway lead to beneficial effects on the clinical manifestation (Hu et al., 2012), Moreover, this system is being explored as potential biomarker for the disease progression. Accordingly, C3 levels were found to be correlated with the disability score (Tatomir et al., 2017). However, accordingly with what we observed in our transgenic mice and immunohistochemistry results, the complement deposition and contribution to the pathophysiology of MS occur late in the disease (Michailidou et al., 2017). Several studies are emerging to determine the role of Trem2 phagocytic receptor in MS pathology but little is known so far. We here investigated the effect of its loss of function on the EAE clinical score, as we did for complement KO mice. Trem2 KO did not show any effect on the EAE onset compared to their littermates but surprisingly mice demonstrated to have less severe motor symptoms with a reduced peak and fast recovery. Our findings do not correlate with the previous

studies in which it was demonstrated a potential beneficial anti-inflammatory activity of Trem2 (Takashi et al., 2007). As I mentioned, very little is known about the ligands of Trem2 and their signaling pathway. Konishi and Kiyama in their review have suggested that Trem2 may have an additional signaling protein other than the well-known DAP12. Notably, loss of function of DAP12 led to similar results to ours, i.e. reduction in the peak and disease severity. On the other hand, the inhibition of Trem2 in MS animal model leads to exacerbation of the disease (Konishi et al.,2018). We have induced EAE in N=10 Trem2 KO mice which is not a significant number due the heterogeneity of the clinical symptoms. Therefore, the role of Trem2 and related Trem2 targets and downstream signaling in the inflammatory response in EAE model will be further investigated.

Taken together, microglia in the hippocampus during the presymptomatic phase of EAE was associated with a resting state, and no differences in level of activation or proliferation was found, which might contribute to the hippocampal homeostasis in this stage of the disease. Therefore, further electrophysiological studies including patch-clamp recordings will be used to further investigate the functional role of the increased GABA presynaptic vesicular transporter (Vgat) on the hippocampal glutamatergic and GABAergic transmission, and the effect of its pharmacological inhibition. Additional time points in the EAE will be also studied, since we aim to investigate microglia and classical complement pathway involvement on the hippocampal synaptic pathology during late stage of the disease. Moreover, we will focus on Trem2 and its role on EAE progression.

Microglia, as well as, complement system exert an important role in neuroinflammatory events occurring in MS (Michailidou et al., 2015). Therefore, selectively promoting microglia to the anti-inflammatory phenotype have been proposed as a potential disease-modifying therapeutic strategy for several neurodegenrative diseases including MS (Zhao et., 2020). Complement components do not partecipate on the onset of the disease, thus, represent their limitation as a therapeutic option (Michailidou et al., 2017). Synapses pathology contibributes the pathophysiology and cognitive impairments associated with several neurological disorders such as schizzophrenia (Berdenis et al., 2020), AD (Jackson et al., 2019) and MS, in which was found to occur prior to demyelination and axonal pathology (Friese et al., 2016). Indeed, targeting synaptic dysregulation in MS for example by modulating the glutamate reuptak (Gentile et al., 2018), have showed prominent neuroprotective effect by preventing excitotoxic damages. Therefore, studying the early pathological mechanisms of synaptic dysfunction associated with MS disease, may contribute to identify new potential therapeutical targets to rescue the neuronal networks and contibute to the improvement of cognitive dysfunction.

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