AN INVESTIGATION INTO THE INTERACTION BETWEEN HIPPOCAMPAL CELLS AND LYMPHOCYTES AND ITS INFLUENCE ON HIPPOCAMPAL NETWORK ACTIVITY.

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Abstract.

While the brain is no longer recognized as an immune-privileged site, the role of infiltrating T cells in either homeostasis, protective or pathological conditions is still poorly understood. In the present study, I hypothesize that neuronal-lymphocyte interactions lead to altered hippocampal network activity. Therefore, I aim to investigate contact-dependent and -independent effect(s) of lymphocytes on neuronal and astrocytic activity and synaptic communication.

Mouse primary hippocampal cultures were prepared on coverslips and in microfluidic devices. Standard Ca^{2+} imaging techniques were used to monitor cellular excitability following treatment (1 hour) with naïve lymphocytes, lymphocyte conditioned medium (LCM) or recombinant IL-16 (rIL-16; 300 pg mL^{-1}). ELISAs confirmed IL-16 in LCM.

LCM but not lymphocyte exposure significantly impaired glutamate-induced increases in neuronal intracellular calcium ($n[Ca^{2+}]_i$) compared to HCM control but both were without effect on KCl-induced elevations in $n[Ca^{2+}]_i$ and ADP- and glutamate-induced astrocytic $[Ca^{2+}]_i$ ($a[Ca^{2+}]_i$) responses. IL-16 levels were elevated in LCM but not during lymphocyte exposure, which indicates that IL-16 is likely to underlie the observed effects with LCM. Interestingly, data has shown a trend towards rIL-16-mediated (300 pg mL^{-1}) impaired glutamate-induced $n[Ca^{2+}]_i$ increases and was without effect on KCl-induced $n[Ca^{2+}]_i$ increases and ADP- and glutamate-induced $a[Ca^{2+}]_i$ responses. As mouse hippocampal neurons and astrocytes do not express CD4 the possible effects observed with LCM and IL-16 treatment were considered CD4-independent. Furthermore, my data shows functional glutamate-induced neuronal synaptic communication between two mouse networks cultured in microfluidic devices. However, contact-dependent lymphocyte effects on glutamate-induced $n[Ca^{2+}]_i$ increases and synaptic communication could not be confirmed in these microfluidic devices. It was shown that lymphocytes do not induce lymphocyte-mediated hippocampal cytotoxicity.

In conclusion, presented data shows that lymphocytes do not mediate hippocampal cytotoxicity and that their soluble mediators, likely to be IL-16, impair hippocampal Ca^{2+} signalling via glutamate receptor modulation.

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List of Abbreviations.

a[Ca2+]i	Astrocytic intracellular calcium
AD	Alzheimer's disease
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine 5′-diphosphate
AF	Alexa Fluor
AMPA	A-amino-3-hydroxy-5-methyl- isoxazole-4-propionate
AMPAR	AMPA receptor
ANOVA	Analysis of variance
AP	Action potentials
APC	Allophycocyanin
APCs	Antigen-presenting cells
APP	Amyloid precursor protein
Ara-C	Cytosine β-D-arabinofuranoside
BBB	Blood-brain-barrier
BCR	B cell receptor
BCSFB	Blood-CSF- barrier
BDNF	Brain-derived neurotrophic factor
BDV	Borna disease virus
BLMB	Blood-leptomeningeal-barrier
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
BPU	Biological Procedures Unit
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CAMKII	Calmodulin-dependent protein kinase II
CAMs	Cell adhesion molecules
CaN	Calcineurin
CAPS	Calcium-dependent activator protein for secretion
CCL19	Chemokine ligand 19
CCR7	C-C chemokine receptor type 7
CDC	Complement-dependent cytotoxicity
CGN	Cerebellar granule neuron
CIITA	Class II transactivator
Cl^-	Chloride
cNB-A	Complete Neurobasal-A medium
CNS	Central nervous system
cRPMI	Complete Roswell Park Memorial Institute
CSF	Cerebrospinal fluid
CTLA	Cytotoxic T lymphocyte antigen
CXCL12	CXC-chemokine ligand 12
DALYs	Disability-adjusted life-years
DAMPs	Damage-associated molecular patterns

DCN	Deep cerebellar nuclei
DC	Dendritic cell
DHPG	Dihydroxyphenylglycine
DI	Deionised
dIL-16	Denatured IL-16
DIV	Days *in vitro*
DMEM	Dulbecco modified Eagle's medium
DR	Death receptor
DRG	Dorsal root ganglion
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPSC	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
Fab	Antigen-binding fragment
FACS	Fluorescence-activated cell sorting
FAD	Familial Alzheimer's disease
FBS	Fetal bovine serum
Fc	Crystallisable fragment
FITC	Fluorescein
FSC	Forward scatter
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GluA	AMPA receptor subunit
GluN	NMDA receptor subunit
Glutamate	L-glutamic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNR	Glia/neuron ratio
HBBS	Hank's balanced salt solution
HCM	Hippocampal conditioned medium
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLCM	Hippocampal lymphocyte conditioned medium
HMGB1	High mobility group box 1
hNSC	Human embryonic/foetal brain-derived neural stem cell
HRP	Horse radish peroxidase
HSC	Hematopoietic stem cells
HSV-1	Herpes simplex virus type 1
I/R	Ischaemia-reperfusion
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T cell costimulator
IFN	Interferon
$[Ca^{2+}]_i$	Intracellular calcium
Ig	Immunoglobulins

iGluR	Ionotropic glutamate receptors
ILC	Innate lymphoid cell
IL	Interleukin
iNOS	Inducible nitric oxide synthase
$InsP_3$	Inositol trisphosphate
IPSP	Inhibitory postsynaptic potential
ISF	Interstitial fluid
ISS	Interstitial system
JAK	Janus kinase
K^+	Potassium
KA	Kainite
KCl	Potassium chloride
$KMeSO_3$	Potassium methanesulfonate
LAK	Lymphokine-activated killer
LCF	Lymphocyte chemoattractant factor
LCM	Lymphocyte conditioned medium
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAC-1	Macrophage-1 antigen
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MgATP	Magnesium adenosine 5′-triphosphate salt
$MgCl_2$	Magnesium chloride
mGluRs	Metabotropic glutamate receptors
$MgSO_4$	Magnesium sulphate
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
$n[Ca^{2+}]_i$	Neuronal intracellular calcium
Na^+	Sodium
NaCl	Sodium chloride
NaGTP	Guanosine 5′-triphosphate sodium salt
NaH_2PO_4	Monosodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	Neurofibrillary tangles
NFU	Normalised fluorescent unit
NIL-16	Neuronal IL-16
NK	Natural killer
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor

NOD	Nucleotide-binding oligomerization domain
NT	Neurotrophin
OGD	Oxygen glucose-deprived
OSC	Organotypic slice culture
P/S	Penicillin-streptomycin
P13K	Phosphoinositide 3-kinase
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD-1	Programmed death receptor 1
PDMS	Polydimethylsiloxane
PerCP	Peridinin Chlorophyll Protein Complex
PFA	Paraformaldehyde
PI	Propidium iodide
PKC	Protein kinase C
PLL	Poly-L-lysine
PLO	Poly-L-Ornithine
PLP	Proteolipid protein
pMHC	Peptide-MHC
PNS	Peripheral nervous system
PRR	Pattern-recognition receptor
PS	Presenilin
rIL-16	Recombinant IL-16
RMP	Resting membrane potential
ROI	Regions of interest
Rorγt	Receptor-related orphan receptor-γ-t
ROS	Reactive oxygen species
-Si-OH	Silanol
SCI	Spinal cord injury
SEMA	Semaphorin
SNARE	Soluble NSF attachment receptor
SSC	Side scatter
STAT	Signal transducer and activator of transcription
Tat	Transactivator of transcription
TCR	T cell receptors
Th	T helper
TLR	Toll-like receptor
tMCAO	Transient middle cerebral artery occlusion
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
VCAM	Vascular adhesion molecules
VLA	Very late antigen
WNV	West Nile Virus

Chapter 1

General introduction.

This thesis is a result of a multidisciplinary approach, including neuroscience, immunology and engineering, to investigate cellular contact-dependent and -independent interaction(s) between the central nervous system (CNS) and immune system. Hence, this chapter presents a review of the current knowledge about the CNS-immune crosstalk, directly relevant to the aims of this thesis, to provide a foundation and motivation for the presented work. Special focus will be given to T cell infiltration in CNS homeostasis and in different CNS diseases as well as their influence on various CNS cell types, including neurons and glia. Furthermore, this chapter describes a novel state-of-the-art technique that can help to better understand underlying mechanisms of the CNS-immune interactions in health and disease.

1.1 General introduction to the CNS.

The nervous system of the human body makes us what we are, an individual with emergent properties, including consciousness, learning, memory as well as feelings and emotions, but it also provides us with locomotor control and sensory perception (Henke, 2010; Damasio and Carvalho, 2013; Froemke *et al.*, 2013; McLean and Dougherty, 2015). This highly organised system is divided into the CNS and peripheral nervous system (PNS). Within the CNS, the brain and spinal cord are the two main components responsible for perceiving and sending out information, but are also involved in storing, recalling and analysing this information. The brain is acknowledged as the control centre and consists of a left and right hemisphere, which can be subdivided into 6 compartments; the cerebrum, diencephalon, midbrain, cerebellum, pons and medulla oblongata. The latter four are collectively termed the brain stem, which is adjoining and structurally connected to the spinal cord that extends down near to the second lumbar vertebrae. Hence, the spinal cord is considered the major conduit for information travelling between the brain and the PNS and vice versa, connecting the CNS with the body's limbs and organs. Information travelling in the CNS and PNS is carried out by signalling neurons and their supporting glial cells (Figure 1.1) that form a complex network as they interact and communicate with one another and/or target tissues.

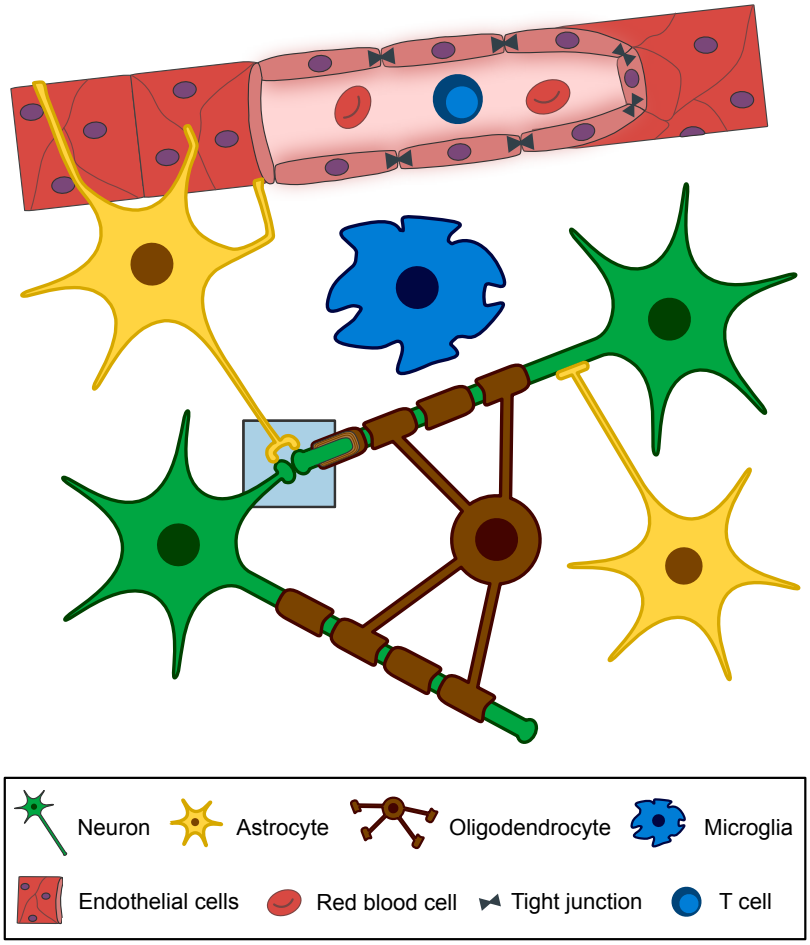

Figure 1.1. Schematic representation of various cell types present in the CNS. Oligodendrocytes form myelin sheaths surrounding neuronal axons, which facilitate neuronal electrical transmission via synapses. In close proximity of these synapses, astrocytes are found forming a tripartite synapse (grey box), promoting neuronal transmission. In addition, they maintain the BBB via endothelial cell interactions, preventing entry of inflammatory cells. Furthermore, neurons communicate with microglia and astrocytes via axons and dendrites.

Glial cells can be further divided into macroglia, including astrocytes and oligodendrocytes, and microglia, which occupy together with the neurons 70-80% of the total brain volume (Fenstermacher and Kaye, 1988; Syková and Nicholson, 2008; Nicholson *et al.*, 2011). Furthermore, the brain is constituted by the vascular (~3%) and interstitial system (ISS, 15-20%). The latter is composed of the interstitial fluid (ISF), which is involved in waste disposal,

and extracellular matrix (Syková and Nicholson, 2008; Lei *et al.*, 2017). The brain's remaining volume is occupied by cerebrospinal fluid (CSF). Originally, it was considered a widespread motion that the glia/neuron ratio (GNR) uniformly increases with brain size and that glial cells represent the most abundant cell type in the brain with a 10:1 ratio (Kandel *et al*., 2000; Paradiso *et al*., 2007). However, it is now accepted that overall the human brain consists of an equal amount of neurons and glia, but the GNR varies across different parts in the brain due to large variation in neuronal cell sizes and lesser variation in glial cell sizes (Azevedo *et al.*, 2009; von Bartheld *et al*., 2016).

As parenchymal CNS cells cannot be easily replaced or repaired, any kind of damage to the brain would be devastating (Silver, *et al*., 2015). The brain is therefore the best protected organ in the body, with multiple protective layers, generating a CNS microenvironment in homeostasis. First, it is protected by a bony structure called the cranium, with underlying meninges, including a thick dura mater and two much thinner layers of arachnoid and pia mater, starting from bone to tissue (Decimo *et al.*, 2012). Veins in the dura mater drain blood from the brain through venous sinuses and the pia mater is associated with arteries that supply blood to the brain and spinal cord (Adeeb *et al.*, 2012, Adeeb *et* al., 2013). The arachnoid mater is loosely attached to the pia mater, creating the subarachnoid space, which is occupied by CSF (Adeeb *et al.*, 2013). CSF is secreted by choroid plexus epithelium, located in the lateral ventricles of the brain. It then circulates via the third and fourth ventricles to the subarachnoid space, before it gets reabsorbed into the systemic circulation through arachnoid villi that extend through the venous sinuses of both hemispheres (Brinker *et al.*, 2014). This reabsorption into the systemic circulation allows for neurotrophic factor distribution into the brain and stabilises brain pH and chemical gradients (Spector, *et al*., 2015). Furthermore, in addition to supplying an excretory pathway serving as waste disposal for CNS solutes, CSF provides buoyancy, so that brain weight is reduced, resulting in less pressure on blood vessels and nerves (Idris, 2014). A final protective layer is the blood-brain-barrier (BBB), consisting

of endothelial cells, astrocyte end-feet and pericytes, which provides a barrier to potentially neurotoxic plasma components, immune cells and pathogens (Obermeier, *et al*., 2013). It also limits hydrophilic molecules from the blood entering the brain but does allow for the exchange of small lipophilic molecules including oxygen (O_2) and carbon dioxide (CO_2), which can then freely cross along their concentration gradients (Grieb *et al.*, 1985; Zlokovic, 2011). The BBB also facilitates transport of nutrients and energy metabolites into the brain, as well as it transports metabolic waste solutes from the ISF into the blood via efflux pumps (Omidi and Barar, 2012; Sanchez-Covarrubias *et al.*, 2014). Together with regulating ion balances in the CNS, the BBB is considered a highly selective semi-permeable border.

1.1.1 Neurons.

Neurons are generated from neuroepithelial cells that arise from the neural tube, which is transformed out of the neuroectoderm during embryonic development (Kintner, 2002; Liu and Zhang, 2011). They are the main information processing cells of the CNS and accomplish this by sending out electrical impulses known as action potentials (APs). There are many different types of neurons including, interneurons, Purkinje, granule, basket, stellate, Golgi and pyramidal cells, all performing specific functions and are classified by their morphology, number of dendrites, connections, axon length and type of neurotransmitter they secrete (Paradiso, *et al*., 2007). The general structure of a neuron, however consists of a cell body, also referred to as the soma, an axon and several dendrites (Figure 1.2). The soma contains a cell nucleus, which synthesizes all proteins and membranes necessary for proper neuronal functioning. Dendrites can also be responsible for this, but their main function is to receive stimuli from other neurons (Kapitein *et al.*, 2010). Whenever a neuron has processed an incoming AP via its dendrite, its electrical signal is transferred to another neuron via its axon. This takes place via synapses between presynaptic axon terminals and postsynaptic dendrites. In other words, it allows neurons to communicate with each other via electrical or chemical signals by means of synaptic transmission (see section 1.2, Trenholm *et al.*, 2014).

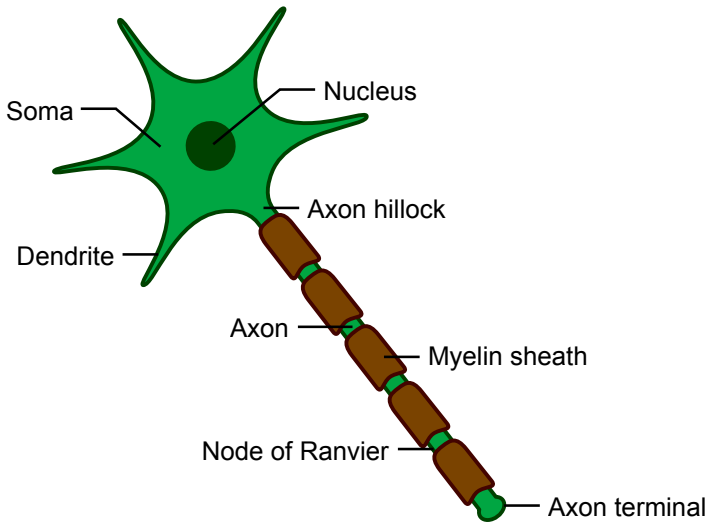

Figure 1.2. Schematic representation of neuron's typical structure.

1.1.2 Oligodendrocytes.

Oligodendrocytes are a type of macroglial cell that form myelin sheaths, a multilayer membrane, which envelope neuronal axons. This membrane sheath consists of 70-80% lipids and 20-30% proteins and is only produced by mature non-proliferating oligodendrocytes (Hu *et al.*, 2004). A single oligodendrocyte can produce 40 myelin segments on multiple neuronal axons (Miron, *et al*., 2011). Unmyelinated segments are called nodes of Ranvier. At these nodes, the neuronal axon's membrane is in direct contact with the extracellular environment, so that ions can be exchanged across the axon membrane through voltage-gated channels (see section 1.2, Arancibia-Carcamo and Attwell, 2014). Therefore, myelin functions as a rapid nerve conduction facilitator, as APs jump from one node of Ranvier to the next, also known as saltatory conduction. Myelin produced by oligodendrocytes also modulates axonal maturation, survival and regenerative capacity through trophic support and signalling molecules (Paz Soldán and Pirko, 2012). When axons become demyelinated, APs cannot jump between nodes of Ranvier and therefore electrical impulse conduction is reduced, preventing neurons from efficient communication. Eventually, this can result in motor and sensory deficits as seen in CNS disorders, including multiple sclerosis (MS, Mahad, *et al*., 2015).

1.1.3 Astrocytes.

Just like neurons, macroglial cells are derived from the neuroepithelium and transformed into radial glial progenitor cells, which then in turn differentiate into neurons and specific macroglial cells (Martynoga, *et al*., 2012). Astrocytes are the most abundant form of macroglial cells in the CNS and can be divided into protoplasmic and fibrous astrocytes. While protoplasmic astrocytes possess highly branched bushy processes and are mainly located in grey matter, fibrous astrocytes possess straight and long processes and are widely distributed in white matter. Whilst they contain sodium and potassium voltage-gated channels (Bevan *et al.*, 1985), which have a crucial role in cell excitability, they are characterized as electrically silent (Vardjan and Zorec, 2015). However, it was shown that astrocytes do exchange information with pre- and post-synaptic neurons, forming a "tripartite synapse" (Figure 1.3, Perea and Araque, 2010). Excitation in astrocytes is not comparable to neuronal excitation, but is displayed in the form of cytosolic calcium (Ca^{2+}) signalling (Wu *et al.*, 2015). Elevations in Ca^{2+} levels can occur spontaneously or upon neurotransmitter release via neuronal synaptic transmission (Eroglu and Barres, 2015). Thus, astrocytes have a primary role in synaptic activity, however they are also involved in ATP production and maintaining homeostasis in extracellular fluid by controlling glycogen storage for glucose production, regulating ion and neurotransmitter levels and producing a variety of growth factors (Gee and Keller, 2005; Sofroniew and Vinters, 2010). Furthermore, they form borders along blood vessels in the CNS, also known as glia limitans, in order to control BBB formation, maintenance, function and repair (Verkhratsky and Butt, 2013). During neuroinflammation and ischemia, two different phenotypes of reactive astrocytes have been discovered, Other than this, astrocytes can become reactive in disease states and take up a pro-inflammatory A1 phenotype or immunosuppressive A2 phenotype (Liddelow and Barres, 2017). A1 reactive astrocytes can exacerbate inflammatory conditions by recruiting inflammatory cells into the CNS as they facilitate the opening of the BBB (Liddelow *et al.*, 2017). In contrast, reactive A2 astrocytes exert anti-

inflammatory functions that maintain and repair the BBB, therefore restrict inflammation and protect neurons as well as promote scar formation (Liddelow *et al.*, 2017). This over-simplistic terminology parallels the M1 and M2 phenotype observed in macrophages and is also applied to microglia (see section 1.1.4). However, as macrophages and microglia can display more than two polarization states, it is still unclear whether astrocytes can adopt more than these two described phenotypes. Nevertheless, astrocytes are considered to play a fundamental immunological role in the healthy and diseased CNS.

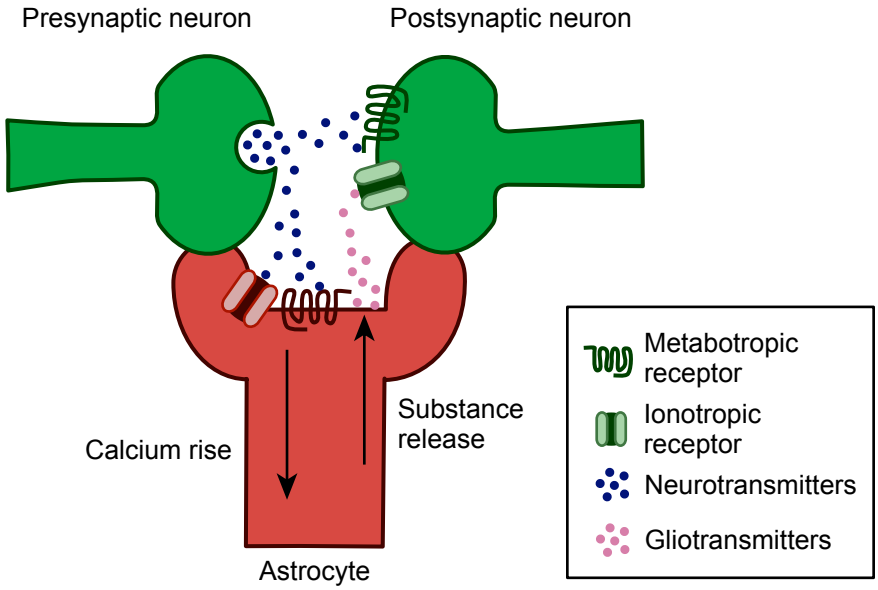

Figure 1.3. Schematic representation of the tripartite synapse. Following neurotransmitter release from presynaptic neurons, astrocytes are activated via receptors, leading to a rise in astrocytic Ca^{2+} and a subsequent gliotransmitter release. These gliotransmitters then act back on both pre- and postsynaptic neurons, enhancing or inhibiting their activity.

1.1.4 Microglia.

In addition to astrocytes and oligodendrocytes, an important subset of glial cells are the microglia, representing ~10% of the total CNS cell population (Domingues *et al.*, 2016). In contrast to neurons and macroglia, these cells are mesodermal, more specifically haematopoietic in origin (Casano and Peri, 2015). They arise from yolk sac primitive

macrophages (Gomez Perdiguero *et al.*, 2014) and maintain themselves via self-renewal in comparison to non-proliferating neurons (Hashimoto *et al.*, 2013). It is well established that microglia are part of the CNS' innate immune system and therefore proposed as specialised immune cells that act as phagocytes, clearing neuronal debris and infectious agents. They also facilitate CNS maintenance by releasing neurotrophic factors, cytokines and chemokines in response to stimulation of their cell membrane receptors and potassium channels via changes in the CNS' specialized microenvironment (Hu *et al.*, 2015). These neurotrophic factors promote neuronal growth and differentiation as well as regulate synaptic plasticity by modifying and eliminating synaptic structures (Hansson and Rönnbäck, 2003). Recently, it was discovered that microglia play a role in synaptic development and maintenance of neuronal interactions by complement system activation (Zabel and Kirsch, 2013).

Once microglia sense a foreign substance or an indication of harm, they enter an activated state in which they take on different phenotypes. This microglial activation depends on the milieu in which they get activated and their stimulating factors (Tang and Le, 2016). Since the CNS microenvironment differs between various parts of the brain, local microglial phenotype is very diverse (Grabert *et al.*, 2016). In general, activation states can be categorized in two main types, including "classical activation" and "alternative activation". With classical activation, so-called M1 phenotype microglia expand tissue damage by releasing a large number of proinflammatory cytokines and chemokines as well as reactive oxygen species (ROS) that are detrimental to surrounding cells (Orihuela, *et al*., 2016). Whilst they also impair CNS repair, this can be counteracted by M2 microglia that are in an alternative activation state (Cherry *et al.*, 2014). These cells take on an anti-inflammatory phenotype that is involved in CNS tissue repair, debris clearance and regeneration. It has to be mentioned thought that the terminology of microglia subsets has been retrieved from macrophage polarization in an attempt to simplify data interpretation at a time when the difference in functionality of microglia was not yet characterized. Hence, thoughtful investigations based on transcriptomic signatures have

shown us that M1/M2 microglia polarization might not exist, as canonical markers of divergent polarization states were co-expressed on single cells, marker gene expression of defined *in vitro* polarization states was at random and was ineffective at predicting presence of other polarization genes (Martinez and Gordon, 2014; Xue *et al.*, 2014; Ransohoff, 2016).

In pathological CNS conditions, an astrocyte and microglial crosstalk has been shown, in which earlier activated microglia are involved in astrocyte activation, or astrocytes facilitate activation or inhibition of distant microglial function (Liu, *et al*., 2011). Additionally, microglia as well as astrocytes contribute to adaptive immune responses by interacting with T lymphocytes (T cells) that have entered the CNS during inflammatory conditions (see section 1.8, Town, *et al.*, 2005; Xie and Yang, 2015).

1.1.5 Vascular endothelial cells.

An active interface between the blood and the CNS is represented by specialized endothelial cells that comprise the vasculature of the BBB. Tight junctions and adherens junctions, which are comprised of transmembrane adhesion molecules including cadherins, occludins and claudins (Berndt *et al.*, 2019), maintain cerebral endothelial cell-to-cell contact by sealing the paracellular cleft. This prevents T cells from entering the CNS. In addition to preserve the BBB, endothelial cells support metabolic homeostasis, coagulation, vascular hemodynamics and permeability, and positive cellular recruitment. Before leukocytes can interact with endothelial cells and thus cells can be recruited into the CNS, endothelial activation is required. This is represented by a loss of vascular integrity, expression of adhesion molecules, an anti-thrombotic-to-prothrombotic phenotype change and cytokine production (Wu et al., 2017). In addition, endothelial cells actively participate in both innate and adaptive immunity and are therefore considered first responders to pathogens (Danese et al., 2007; Al-Soudi et al., 2017; Konradt and Hunter, 2018). As such, cerebral endothelial cells express different receptors related to innate and adaptive immunity, including pattern-recognition receptors (PRRs) such

as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, chemokine receptors and tumour necrosis factor receptors (TNFRs) (Venkatesh *et al.*, 2013; Nagyőszi *et al.*, 2015; Salvador *et al.*, 2016; Haarmann *et al.*, 2019). Upon activation of these receptors, tight junctions are disrupted, and endothelial cells produce inflammatory cytokines mediating leukocyte infiltration (see section 1.8).

1.2 Synaptic transmission.

1.2.1 Action potential generation and propagation in neurons.

Any cognitive or motor tasks as well as the perception of sensory stimuli requires communication between neurons via APs. During an AP, a neuron's membrane potential rapidly rises and falls (Figure 1.4 A). Initiation and propagation of these APs relies on in- and outward flows of certain ions controlled by various non-, ligand- and voltage-gated ion channels (Hille, 2001). Therefore, defects in synaptic communication between neurons can occur when channels are improperly distributed (Lai and Jan, 2006).

When a neuron is at rest and not generating any APs, it maintains a stable resting membrane potential (RMP) of -60 to -70 mV (Figure 1.4 Phase 1, Squire *et al.*, 2012). This results from the uneven but stable distribution of ions, including sodium (Na^+), potassium (K^+), Ca^{2+} and chloride (Cl^-), situated in the intra- and extracellular fluid around the cell membrane (Table 1.1), leading to a neuronal membrane potential that is negatively charged on the inside and positively charged on the outside. To maintain this stable ion distribution, various transporters actively transport ions across the plasma membrane, including the Na^+/K^+ pump, counteracting the flow of Na^+ and K^+ ions via leak channels, and thereby maintaining Na^+ and K^+ gradients. K^+ ions are the major contributor of the RMP, as K^+ is the ion that is closest to being in electrochemical equilibrium when the cell is at rest (McCormick, 2014). Any changes in either the ions' concentration gradients or membrane permeability due to a stimulus from the environment or another neuron will alter the RMP.

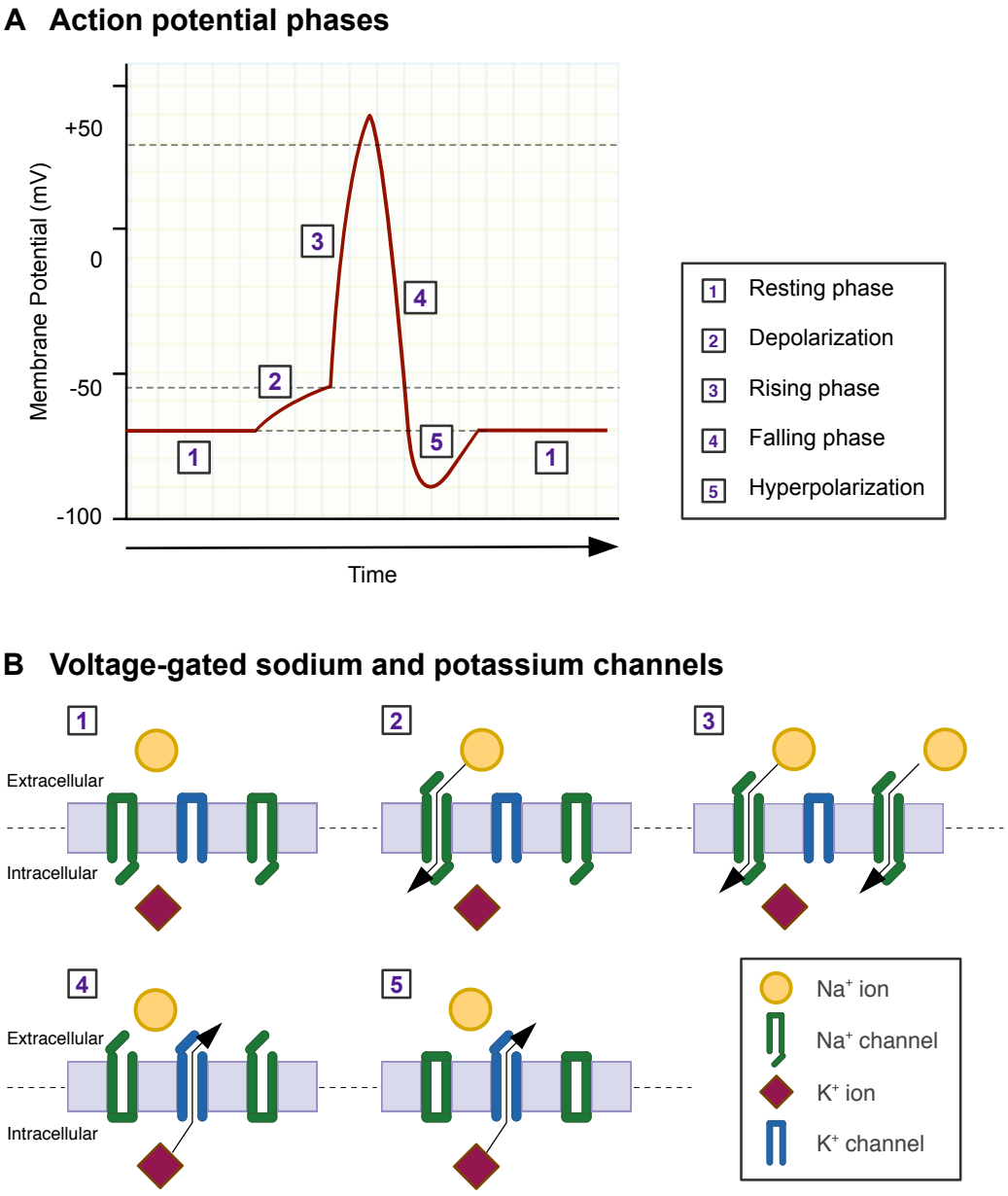

Figure 1.4. Schematic representation of Na^+ and K^+ crossing the neuronal membrane during action potentials. A) Changes in membrane potential during an AP. B) Na^+ and K^+ in- and outward flow while voltage-gated sodium and potassium channels open and close. Phase 1) Sodium and potassium channels are closed during RMP (-70 mV). Phase 2) Membrane depolarizes to threshold and sodium channels open. Phase 3) Depolarization passes the threshold and more sodium channels open, causing a rapid Na^+ entry. Phase 4) Potassium channels open and K^+ flows out the cell, leading to repolarization. Phase 5) Potassium channels remain open, causing the cell to hyperpolarize, before it goes back to its RMP.

Table 1.1. Extra- and intracellular concentrations of various neuronal ions.

Ion	Extracellular concentration (mM)	Intracellular concentration (mM)
Cations		
Na^+	150	5
K^+	5	100
Ca^{2+}	2	0.0002
Anions		
Cl^-	150	3
A^- fixed anions	–	385

The rising phase of an AP is called depolarization (Figure 1.4 Phase 3) and only happens when the cell's membrane potential passes the excitation threshold of -55 mV (Platkiewicz and Brette, 2010). Therefore, APs are an all-or-none response. Before the membrane potential reaches this threshold, only a few voltage-gated sodium channels are activated (Figure 1.4 Phase 2). When the membrane potential has passed this threshold, more voltage-gated sodium channels are activated, and the membrane becomes even more permeable to Na^+ (Figure 1.4 Phase 3). Depolarization continues until the inside of the cell has become more positive than the outside. At this moment, voltage-gated sodium channels close, which is regulated by inactivation gates located at the intracellular side of the channel and activation gates at the extracellular side of the channel who regulate the opening of voltage-gated sodium channels (McCormick, 2014). The closure of inactivation gates of voltage-gated sodium channels prevents further depolarization and initiates repolarization as the membrane potential has already become more permeable to K^+ due to voltage-gated potassium channel opening, allowing the efflux of K^+. As potassium channels open much slower than sodium channels, they are therefore already fully open by the time depolarization stops and the AP has reached its peak. As K^+ moves out of the cell down its concentration gradient, the membrane potential becomes less positive and returns to its RMP, also known as the falling phase (Figure 1.4 Phase 4). Because of the equilibrium potential of K^+ (-90 mV) being lower than the RMP,

voltage-gated potassium channels and leak potassium channels stay open and K^+ keeps flowing out the cell, eventually leading to membrane hyperpolarization (Figure 1.4 Phase 5, Hille, 2001). Hyperpolarization is also due to the slow inactivation of voltage-gated potassium channels (Pless *et al.*, 2013). While the potassium channels close, Na^+/K^+ pumps ensure that the membrane potential returns back to -70 mV by actively exchanging three intracellular Na^+ ions for two extracellular K^+ ions (Morth *et al.*, 2011).

1.2.2 Chemical synaptic transmission.

When APs arrive at the nerve terminal, a neuron either converts its electrical signal into a chemical signal allowing electrical signals to be passed onto the next neuron or they are directly transferred to a neighbouring neuron, a mechanism called synaptic transmission (Pereda, 2014). Proper synaptic communication between neurons depends on multiple factors, including neuron-secreted signalling molecules, target cell receptors on postsynaptic cells and anatomical connections (Silverthorn, *et al*., 2009). Synaptic transmission takes place at synapses that are formed between neighbouring neurons. The vast majority of communication occurs via chemical transmission, due to a requisite for diversity and fine-tuning of synaptic transmission for complex integrated processes that exist in higher organisms (Hormuzdi *et al.*, 2004). Chemical synapses make this possible via presynaptic release of various neurotransmitters and the use of different neurotransmitter receptors on the surface of postsynaptic cells (Figure 1.5). In response to depolarization of an AP in the presynaptic axon terminal, Ca^{2+} enters through the opening of voltage-gated calcium channels (Figure 1.5 Phase 1 and 2). This influx of Ca^{2+} will trigger vesicle discharge of neurotransmitters into the synaptic cleft through binding to regulatory proteins (Südhof, 2014). These neurotransmitters are synthesised in the nerve terminal and then stored in vesicles until required (Figure 1.5 Phase 3). These vesicles are either closely situated to the membrane at the docking site and are readily releasable or they serve as a reserve pool that restores the readily releasable vesicular pool (Südhof, 2013). Vesicle discharge takes then place via docking and fusion mechanisms

during endocytosis (Figure 1.5 Phase 4). Soluble NSF attachment receptor (SNARE) complexes are the most important proteins involved in drawing vesicles to target membranes, described as docking mechanisms, and eventual induction of fusion mechanisms (Zhou *et al.*, 2015). After a vesicle has fused with the cell membrane, fused areas open and neurotransmitters are released in quanta (Isaacson and Walmsley, 1995; Augustine and Kasai, 2007). In specific, each vesicle contains a minimal amount of neurotransmitters necessary to change the membrane potential of a postsynaptic neuron. Additional regulators such as synapsin, synaptotagmin and calcium-dependent activator protein for secretion (CAPS) increase the precision of exocytosis at the synapse (Zhou *et al.*, 2015; Petrie *et al.*, 2016). Subsequently, neurotransmitters travel through the synaptic cleft and bind to their corresponding neurotransmitter receptor on a postsynaptic cell, generating a postsynaptic potential (PSP, Figure 1.5 Phase 5 and 6).

A PSP can either be excitatory or inhibitory (EPSP; IPSP), depending on whether they drive a cell towards a point above or below its firing threshold by the opening or closing of ion channels. Glutamate is the main excitatory neurotransmitter in the CNS with γ-aminobutyric acid (GABA) being the primary inhibitory neurotransmitter (Walls *et al.*, 2015). Glutamate can bind to two types of neurotransmitter receptors on the postsynaptic neuron, including ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors (iGluR) are transmembrane ligand-gated ion channels which allow passage of ions after glutamate binding (Traynelis *et al.*, 2010). These receptors can be divided into N-methyl-D-aspartate (NMDA) receptors, which have the highest affinity for glutamate, a-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors. NMDA receptors are calcium entry channels and directly regulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) release, while AMPA- and KA-receptors indirectly contribute to the entry of Ca^{2+} via NMDA-receptors and voltage-gated calcium channels by cell membrane depolarization (Kononov, *et al*., 2011).

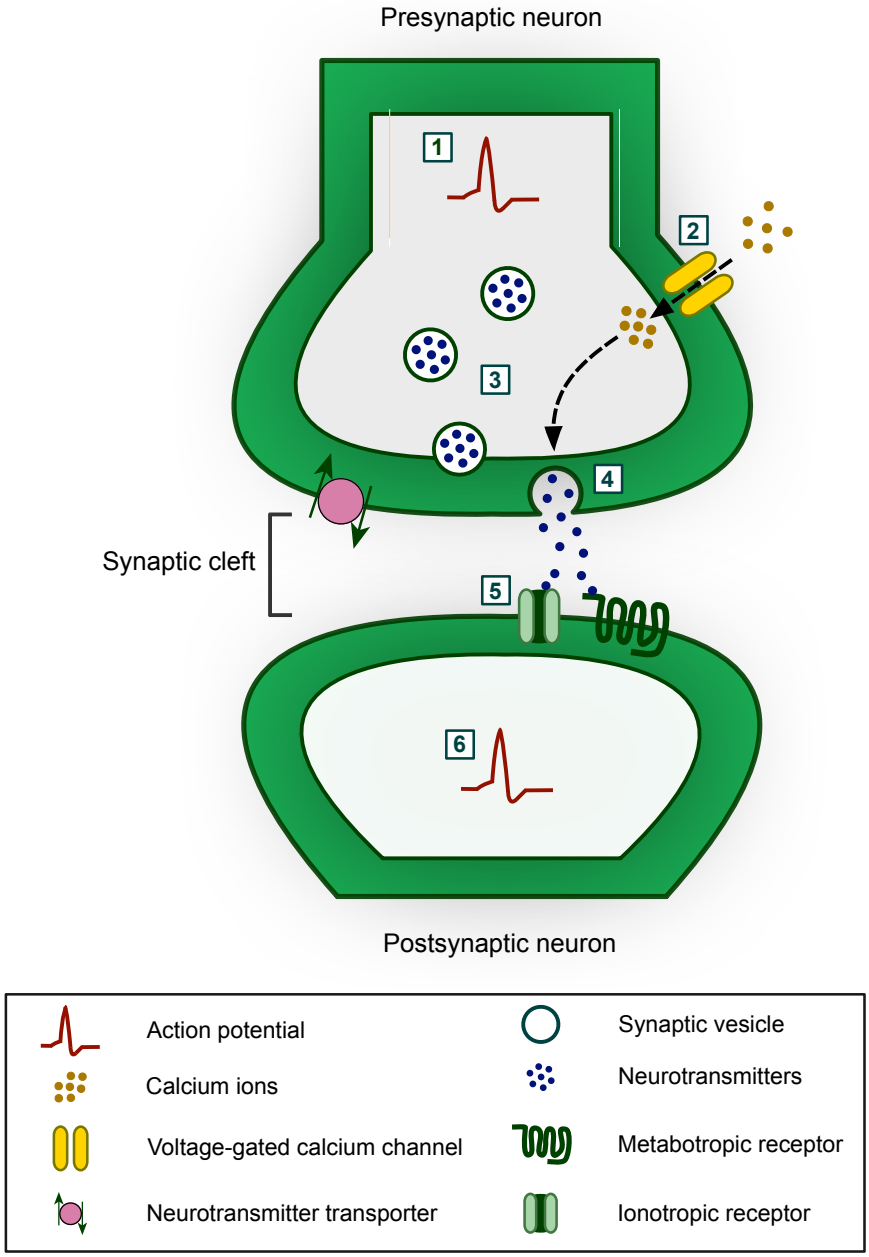

Figure 1.5. Schematic representation of synaptic transmission at chemical synapses. (1) APs depolarize presynaptic axon terminals. (2) Voltage-gated calcium channels open, causing a Ca^{2+} influx. (3) Ca^{2+} entry triggers exocytosis of synaptic vesicle contents. (4) Neurotransmitters get released and diffuse across the synaptic cleft. (5) Neurotransmitters bind to metabotropic or ionotropic receptors on postsynaptic cell. (6) APs are initiated in postsynaptic cell.

In contrast to ionotropic glutamate receptors, metabotropic glutamate receptors (mGluRs) are both found pre- and postsynaptically and belong to the G-protein-coupled receptor superfamily that is known to activate a whole cascade of intracellular signalling partners (Niswender and Conn, 2010). This type of glutamate receptor can be divided into three groups based on sequence homology, G-protein coupling, and ligand selectivity; group 1 mGluRs (mGlu1 & mGlu5), group 2 mGluRs (mGlu2 & mGlu3), and group 3 mGluRs (mGlu4, mGlu6, mGlu7 & mGlu8).

To prevent constant stimulation of postsynaptic cells by continuous neurotransmitter binding to their corresponding receptors, neurotransmitter activity requires to be terminated. This can either be achieved by enzymes that inactivate neurotransmitters (Todorov *et al.*, 1997) or neurotransmitter transporter-induced reuptake into the presynaptic cell (Andersen *et al.*, 2016). Glutamate inactivation for instance can occur via astrocytic reuptake, converting glutamate into glutamine (Shen, 2013) or glutamate transporter-induced reuptake into the postsynaptic or presynaptic cell (Grewer *et al.*, 2008). Similarly, GABAergic activity is terminated by neuronal and astrocytic reuptake via Na^+- and Cl^--dependent transport processes (Hertz and Rodrigues, 2014), as is the case for many other neurotransmitters. Although, some neurotransmitters simply diffuse out of the synaptic cleft, however this can lead to significant receptor activation of neighbouring synapses (Rusakov, *et al*., 1999; van Bommel and Mikhaylova, 2016).

1.2.3 Electrical synaptic transmission.

When electrical signals are directly transferred to a neighbouring neuron, APs in the presynaptic cell induce a passive current flow into the postsynaptic cell, which then propagates the stimulus. Since this current can move freely, this communication can be bi-directional and takes place through gap junctions (Hormuzdi *et al.*, 2004; Song *et al.*, 2016). These gap junctions are little pores, spanning both the presynaptic and the postsynaptic membrane and

serve as conduits between the cytoplasm of two adjoining cells (Pereda *et al.*, 2013). Occurring synaptic transmission allows for rapid transfer of electrical current into the next neuron. Once sufficient enough to generate depolarization above the aforementioned threshold, APs are passed onto the next cell by opening of voltage-gated sodium channels. Although electrical transmission is considered a rapid transfer of electrical signals, it has been thought that electrical transmission does not provide learning as opposed to chemical transmission (Yang, *et al.*, 1990).

1.3 The CNS is linked with the immune system.

As previously mentioned, the CNS is protected from insults or harmful immune cell infiltration via different mechanical and functional mechanisms, including the skull, meninges and BBB. Therefore, both systems have been considered two separate systems for a long time (also see section 1.6). However, research has shown us that the brain communicates with the immune system and vice versa, and therefore it is widely recognised that the CNS and immune system are not two separate organs at all (Chavan, Pavlov and Tracey, 2017; Veiga-Fernandes and Artis, 2018). Mechanisms involved in this bi-directional communication are a crosstalk between the neuroendocrine system and the immune system (Ashley and Demas, 2017) (Figure 1.6). As such, glucocorticoid hormones including cortisol, secreted from the adrenal cortex, circulate via the blood to immune cells. Furthermore, sympathetic innervation of lymphoid organs promotes neurotransmitter and neuropeptide release including norepinephrine and epinephrine. In addition to a CNS output, immune cells communicate with CNS cells via cytokine release, and then cross the BBB via active and passive transport. Release of IL-1β in particular can also stimulate the parasympathetic vagus.

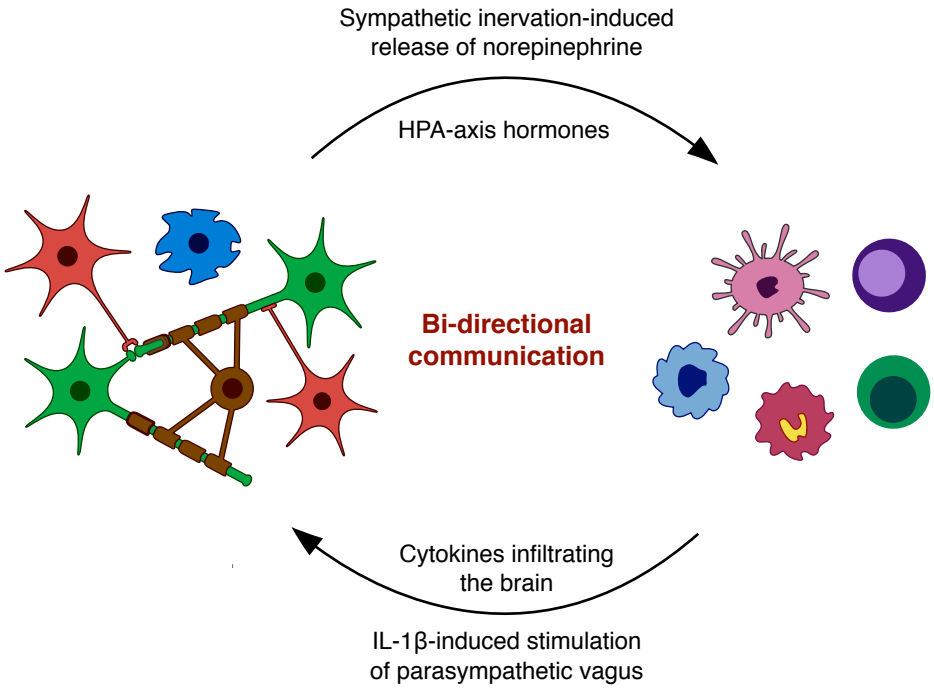

Figure 1.6. Schematic representation of a bi-directional communication between the CNS and the immune system. Signalling between the CNS and the immune system and vice versa both occurs via neural and humoral pathways.

The neuroendocrine-immune crosstalk is believed to contribute to physiological homeostasis by the formation of a negative feedback loop (Gaillard, 2003; Taub, 2008; Ashley and Demas, 2017). However, this communication can also lead to harmful disease states. As such, a modified psychological status results in changes in disease susceptibility and progression of immune-related diseases including cancer, infectious disease and autoimmune disorders (Lasselin, *et al.*, 2016; Watson *et al.*, 2016). In contrast, the CNS can receive feedback from the immune system, inducing sickness behaviour-associated symptoms such as memory impairments, pain stimuli, depression, and fatigue (Abulkassim *et al.*, 2016). The fact that the CNS and immune system share common ligands and receptors further supports the perception of an intra- and inter-system network of communication (Taub, 2008; Guyon, 2014).

1.4 General introduction to the immune system.

Almost all tissues in the body including the CNS can encounter potentially dangerous pathogens and insults. Therefore, the human immune system has evolved to protect the body from damage caused by endogenous and exogenous inflammatory stimuli by producing defence mechanisms referred to as protective immune responses. Exogenous stimuli including exposure to pathogens, allergens or toxins, cause an infectious inflammatory state. In contrast, burns, chemical insults, radiation or oxygen deprivation are considered to contribute to endogenous stimuli that get released by stressed cells or upon tissue damage via necrosis, causing a non-infectious or so-called sterile immunopathology (Gadani *et al.*, 2015). Immune responses that occur following exposure to these stimuli can be divided into innate immunity, frontline of immune defence mechanisms, or adaptive immunity, a more specific immune response with immunological memory (Figure 1.7). Both systems protect the human body, while not responding to self-molecules. However, the immune system can also attack self-tissues, resulting in autoimmune diseases due to an impaired immunological self-tolerance, a state in which the immune system is unresponsive to substances or tissues (Abramson and Husebye, 2016). Autoimmune disease development highly depends on genetics, but also infections, chemical exposure and/or environmental predisposing factors (Farh *et al.*, 2015).

Not only protective but also harmful immune responses caused by the failure of regulating inflammation effectively are mediated by immune cells originating from pluripotent hematopoietic stem cells (HSC) in the bone marrow (BM), which are considered as progenitor or precursor cells, including myeloid progenitor cells and lymphoid progenitor cells (Eaves, 2015). Myeloid progenitor cells are precursors for macrophages, dendritic cells (DCs), mast cells and granulocytes, with the latter being subdivided in neutrophils, eosinophils, and basophils. All these cells are primarily involved in innate immunity and each organ has a unique set of cells that orchestrates this type of immunity.

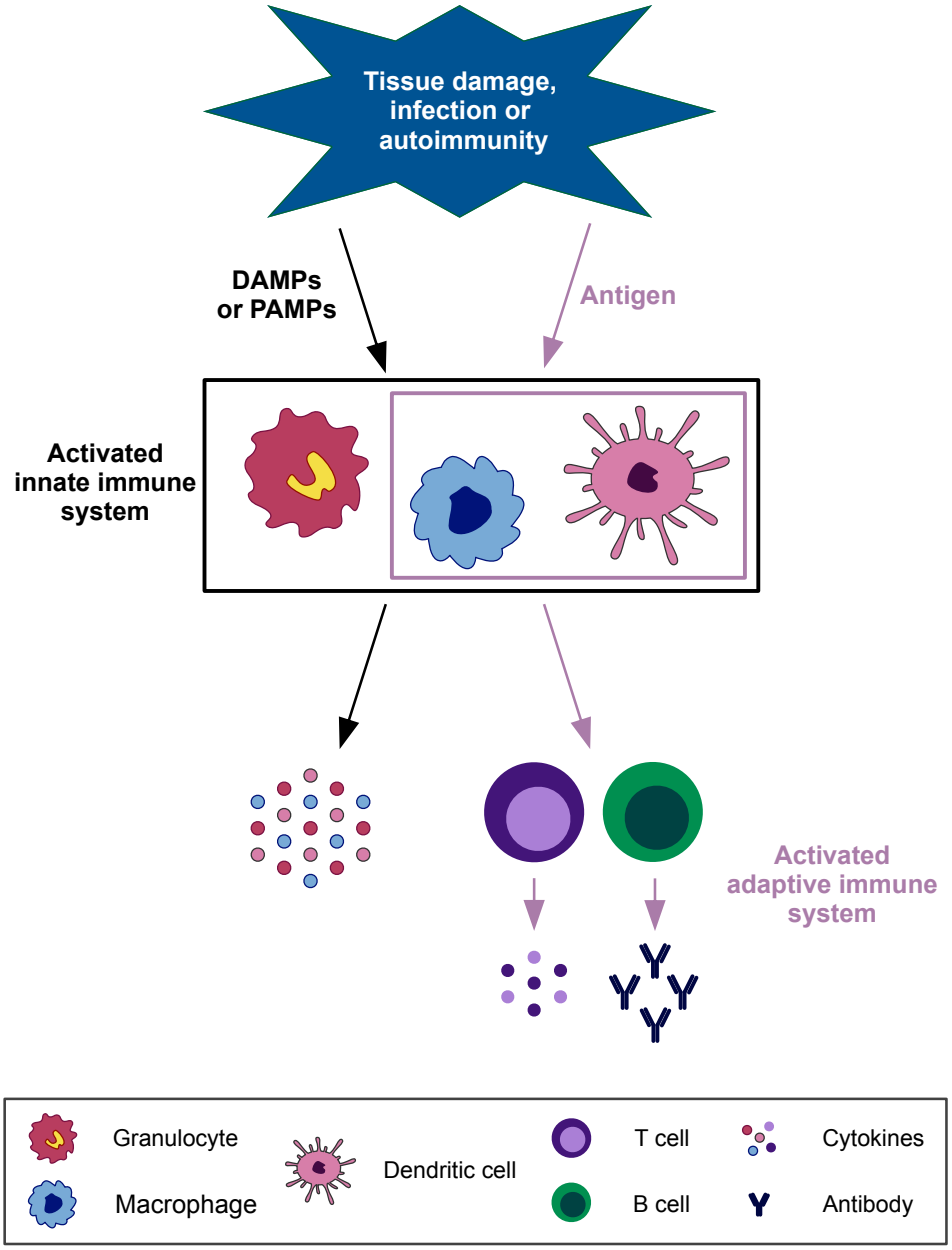

Figure 1.7. Schematic representation of the innate and adaptive immune system. Following tissue damage, infection or autoimmunity, DAMPs, PAMPs or antigen activate granulocytes, macrophages and DCs of the innate immune system. Once activated, they produce pro- and anti-inflammatory cytokines (⟶) or macrophages and DCs present antigen to T and B cells in order to produce cytokines and antibodies, respectively, and thereby activate the adaptive immune system (⟶).

Lymphoid progenitor cells can differentiate into lymphocytes (T and B cells), characteristic of adaptive immunity and innate lymphoid cells (ILCs), belonging to the innate immune system. These innate lymphoid cells lack antigen receptors and can be further divided into natural killer (NK) cells (ILC1), retinoic acid receptor-related orphan receptor-γ-t (Rorγt)-dependent ILCs (ILC3) and type 2 ILCs (ILC2) (Spits, *et al.*, 2016; Vivier *et al.*, 2018). In autoimmune diseases, the most characteristic cells are the antigen-presenting cells (APCs), auto-reactive T cells and auto-antibody-producing B cells (Raphael *et al.*, 2015; Musette and Bouaziz, 2018). To orchestrate an immune response, all these cells communicate with each other via cytokines as well as direct cell-cell interactions. Cytokines, including chemokines, interferons (IFNs), interleukins (ILs), lymphokines and tumour necrosis factors (TNFs) are released upon immunoglobulin- or complement receptor-mediated signalling or pathogen-binding to cellular receptors (Turner *et al.*, 2014). Their release is a complex and tightly controlled process and is different for each cytokine and varies per cell type. As such, classical secretory pathways are primarily mediated by exocytosis, while nonclassical secretory pathways involve membrane transporters, exosomes, microvesicle shedding and cell lysis (Lacy and Stow, 2011).

1.4.1 The innate immune system.

The innate immune system is the host's immediate line of defence against tissue injury (Rani *et al.*, 2017) and invading pathogens, the latter either causing an infection (Erwig and Gow, 2016) or in some cases autoimmunity (Grayson and Kaplan, 2016). This immune mechanism is initiated in different phases, including the innate phase, in which antimicrobial enzymes and peptides together with plasma proteins, known as the complement system, target non-self-pathogens to kill or weaken its effectiveness through cell lysis and phagocytosis (Murphy, 2011). In the accompanying early-induced innate response phase so-called alarmins, including exogenous pathogen-associated molecular patterns (PAMPs) or endogenous damage-associated molecular patterns (DAMPs) are released to alert the host to intruding pathogens or

tissue damage, respectively. These alarmins get recognised by PRRs, primarily TLRs, expressed on the surface of immune cells (Rivera *et al.*, 2016). Bacterial lipopolysaccharide (LPS) is considered the prototypical class of PAMPs, next to flagellin, lipoproteins and nucleic acid variants associated with viruses, while heat-shock proteins, altered membrane phospholipids, uric acid and the high mobility group box 1 (HMGB1) protein belong to the DAMP family (Tang *et al.*, 2012). This immune recognition will lead to an inflammatory response that recruits new phagocytic cells. Phagocytes, including macrophages, monocytes, granulocytes and DCs involved in the first phase also initiate recognition of these pathogen-derived PAMPs and host-derived DAMPs by their PRRs. These PRRs are either present on the cell surface or within intracellular compartments and provide an initial discrimination between self- and non-self-molecules (Thompson *et al.*, 2011). Ligation of PRRs on immune cells has two main advantages to the human body (Suresh and Mosser, 2013), including the detection of 'danger' signals that trigger immune cells to produce cytokines, chemokines and antimicrobial compounds to directly kill the pathogen. These cytokines also initiate an acute phase response that provides the body with several acute-phase proteins produced by the liver, minimizing tissue damage and promoting tissue repair at the same time (Baumann and Gauldie, 1990; Jain, *et al.*, 2011). In addition, antigen-specific adaptive immune responses by cytokine-induced mobilization of APCs, including macrophages and DCs are initiated and introduce the next phase (see section 1.3.2). In case of autoimmune diseases, overt stimulation of innate APCs by genetic defects can break immune tolerance and induce priming of self-reactive T cells, introducing antigen-specific adaptive immune responses. This last advantage provides evidence that the innate and adaptive immune system are linked and not two separate, parallel systems (Sun, Ugolini and Vivier, 2014; Iwasaki and Medzhitov, 2015; Panda and Ding, 2015).

Ultimately, the innate immune system recognizes only a limited number of pathogen- or tissue injury-derived identifying substances. However, these are present on many different agents

and damaged or stressed cells. This system therefore reacts equally adequate to a variety of organisms but is not as specific as the adaptive immune system. Furthermore, it induces an immediate response, as it does not have to be 'learned' through exposure. Hence, proteins and phagocytes involved in innate immunity become quickly activated to destroy inflammatory stimuli. In contrast to the adaptive immune system, innate immunity is not as versatile, and is generally accepted to not have a memory component that provides protection against re-exposure to inflammatory stimuli. However, opposed to the long-lived and highly specific adaptive memory, innate immune cells including monocytes and macrophages have been shown to exhibit a memory phenotype, however this is considered a non-specific short-lived phenomenon (Boraschi and Italiani, 2018). Recently, innate memory has also been shown in microglia as these cells can be pre-activated or "primed" by an inflammatory response, resulting in an amplified secondary response (Neher and Cunningham, 2019).

1.4.2 The adaptive immune system.

For the body to effectively fight a wide range of exogenous and endogenous inflammatory stimuli, it requires an immune response beyond the innate immune system that is only capable of recognising certain molecular patterns or pathogens and producing nonspecific responses. In contrast, adaptive immune responses act with more specificity, achieved by lymphocyte-mediated recognition of certain antigens (Eisen and Chakraborty, 2010). These antigens are either a foreign substance from the environment, including chemicals, bacteria, viruses or pollen, or they may be formed inside the body, such as bacterial toxins or tissue cells. Therefore, likewise the innate immune system, the adaptive immune system is capable of distinguishing between cells of the body itself and pathogens by so-called self-antigens or non-self-antigens. While PRRs recognizing these self-antigens or non-self-antigens involved in innate immunity are encoded in their functional form in the germline genome, receptors of the adaptive immune system are generated by antigen receptor gene assembly (Alt *et al.*, 1992; Carmona and Schatz, 2017). This is because the variability in lymphocyte antigen receptors,

in the form of immunoglobulins (Ig) on B cells and T cell receptors (TCR) on T cells, is immense and therefore not every receptor-chain of a receptor can be encoded in full in the genome. Successful gene rearrangements monitored during lymphocyte development lead to different receptor chain production, creating various functional receptors when combined (Suna *et al.*, 2015). These functional receptors then recognize self-antigens, in case of autoimmunity, or non-self-antigens, in case of infections, directly or indirectly via APCs. Following an antigen encounter, lymphocytes will differentiate into effector cells or into memory cells, which are responsible for long-lasting immunity following exposure to disease or vaccination (Farber *et al.*, 2016). However, in some cases lymphocytes can become functionally inactivated, a state referred to as anergy. This mechanism of tolerance in which lymphocytes remain alive but in a less responsive state can be divided into two main categories (Schwartz, 2003; Mueller, 2010). With clonal anergy, lymphocytes are in a growth arrest state which can be reversed, while with adaptive tolerance, proliferation and effector functions are inhibited due to the absence of co-stimulation.

1.5 Lymphocytes.

The two main lymphocyte classes, T and B cells, are both derived from lymphoid progenitor cells originated in the BM. Whereas B cells mature in the BM, T cell precursors migrate to the thymus to complete their development and become mature T cells. Both T and B cell development into mature cells is defined by the successful expression of antigen receptor gene assembly, with each individual lymphocyte expressing a unique antigen-specific receptor.

1.5.1 T cells.

T cells are a class of lymphocytes that can be divided into CD4 T helper (Th) cells or cytotoxic CD8 T cells. These subsets are determined during maturation when the different cell-surface proteins CD4 and CD8 are upregulated next to the general TCR-CD3 complex that is involved in T cell activation signalling (Ryan, 2010). Once differentiated into its effector cell following

activation, both subsets mediate cellular immunity processes by releasing either inflammatory mediators called cytokines or cytotoxic proteins, including perforin and granzymes (see section 1.5.1.2/3). This cytokine or cytotoxin release depends on the T cell subset, being CD4 or CD8 respectively.

1.5.1.1 T cell activation

Before becoming effector cells, both naïve CD4 and CD8 T cells require activation in peripheral lymphoid tissues via antigen recognition mechanisms (Figure 1.8). Naïve T cells recognize antigens in the form of short peptide fragments presented by major histocompatibility complex (MHC) molecules on the surface of APCs forming an immunological synapse (Sela-Culang *et al*., 2013). This antigen recognition by naïve T cells takes place in two steps, antigen capture/processing by APCs followed by antigen presentation to naïve T cells. Hence, APCs first capture protein antigens by receptor-mediated endocytosis, phagocytosis and pinocytosis, and form short peptide fragments by degrading these internalized proteins through proteolysis (Blum *et al*., 2013). These short peptide sequences are then loaded into the peptide-binding groove of MHC molecules, forming a peptide-MHC (pMHC) complex, which is then presented on the surface of APCs, where they can be scanned by naïve T cells via their TCR. The MHC system's gene products can be divided into MHC class I and class II cell membrane glycoproteins and thereby provide two distinct pathways for the peptide presentation to naïve CD8 or CD4 T cells, respectively (Jensen, 2007). The difference between the two MHC classes does not lie in their structure, but in the source of the peptides that they trap and carry to the cell surface. While MHC class I membrane glycoproteins collect peptides from intracellular antigens, including viruses and some bacterial proteins (Hewitt, 2003), MHC class II molecules bind peptides derived from extracellular proteins that are largely acquired through phagocytic mechanisms (ten Broeke *et al*., 2013).

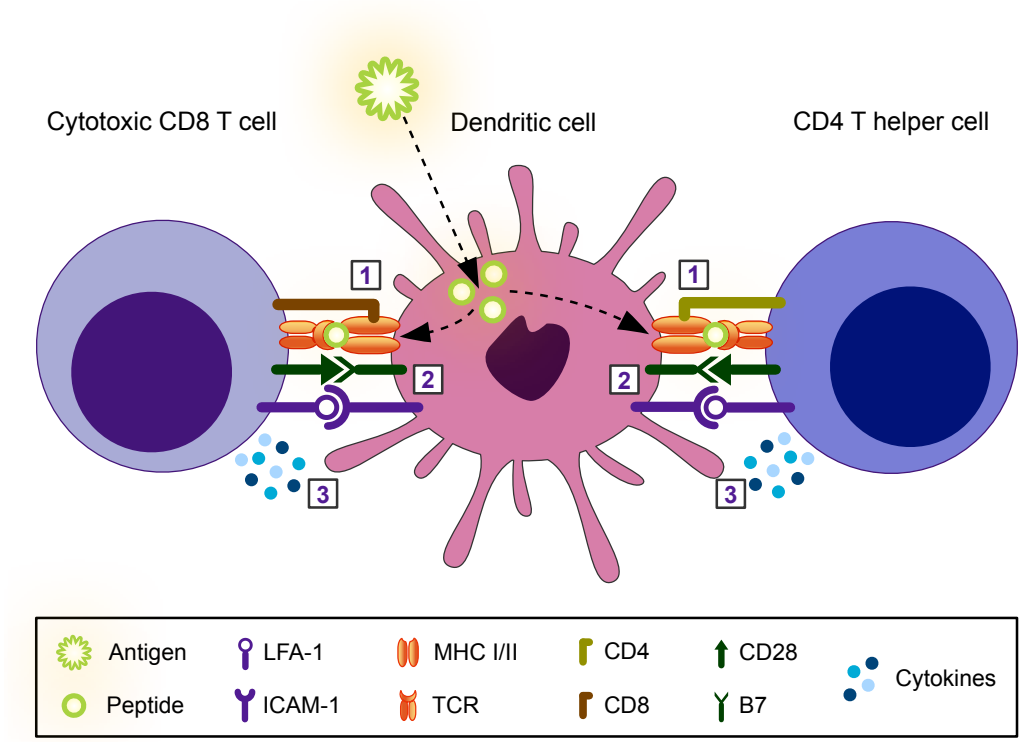

Figure 1.8. Schematic representation of antigen presentation-induced CD8 and CD4 T cell activation. Antigen is taken up by an antigen presenting cell and degraded into short peptides which are then loaded onto the peptide-binding groove of either an MHC I or MHC II molecule. (1) This formed pMHC complex is then presented to the TCR of either a CD8 or CD4 T cells, respectively. (2) Effective activation also requires the display of costimulatory proteins and cell-cell adhesion molecules, including the CD28/B7 complex and the LFA-1/ICAM-1 complex. (3) Cytokines then mediate T cell differentiation.

Thus, as viruses can infect all cells with DNA-replicating capacity, all nucleated cells express MHC class I molecules. APCs that are capable of expressing only MHC I molecules are called non-professional APCs. However, some of these non-professional APCs also express MHC II molecules under inflammatory conditions, including astrocytes (see section 1.9.2). In contrast, APCs that can express both MHC I and MHC II molecules are called professional APCs and include DCs, macrophages and B cells, as well as microglia in the CNS (see section 1.9.1). Under some circumstances, DCs and macrophages can also present exogenous antigens into MHC class I molecules, a process called antigen cross-presentation (Guermonprez and

Amigorena, 2005; Shen and Rock, 2006). Professional APCs can activate naïve T cells, but only when costimulatory proteins and cell-cell adhesion molecules are present (Corthay, 2006). One of the most dominant costimulatory proteins is B7, present on APCs, that interacts with CD28 on T cells (Lenschow, *et al*., 1996). Together with the constitutively expressed B7 molecule, the costimulatory molecules inducible T cell costimulator (ICOS) and OX40 are only expressed by APCs which have encountered a pathogen. Thus, whenever an APC has not encountered a pathogen and still is in a non-activated state, these two costimulatory molecules are not expressed on the cell surface and therefore when a T cell binds to a non-activated APC, the T cell becomes anergic. This then prevents constitutive activation of T cells. In other words, with this second signal involved in T cell activation, erroneous binding can be distinguished from real pathogenic recognition. A second signal can also be introduced via the adhesion molecule lymphocyte function-associated antigen (LFA-1) expressed on the surface of APCs, a ligand for intercellular adhesion molecule 1 (ICAM-1) (Bierer and Burakoff, 1988). Once a T cell is activated in peripheral lymphoid tissues (spleen and lymph nodes), it re-enters the cell cycle and starts to rapidly proliferate in order to differentiate into its effector T cell that is able to produce all the molecules required for their specific helper or cytotoxic functions (Murphy, 2011). This proliferation and polarization of a T cell is mainly mediated by the cytokine IL-2, which is produced by the T cell itself following activation (Liao, *et al*., 2013). Other cytokines derived from innate immune cells, including APCs, the inflammatory environment of the activation site or a cross-talk with other adaptive immune cells further mediate this differentiation process. Whilst it has been long debated where the activation of CNS reactive T cells takes place, it is now widely accepted that naïve T cells bearing auto-reactive TCRs are activated in the peripheral lymphoid tissues, particularly in the gut. In here, cells from the lymphatic system are exposed to epithelial surface-secreted microorganisms and metabolites that facilitates a CD4 Th17 cell type (Ivanov *et al.*, 2008; Berer *et al.*, 2011). Furthermore, gut-derived products are also known to be a natural adjuvant for the

differentiation into autoreactive T cells that have regulatory properties (Hall *et al.*, 2008; Atarashi *et al.*, 2011).

1.5.1.2 CD4 T helper cells.

Activated CD4 T cells differentiate into several subsets of CD4 Th cells with a variety of different functions, including assisting antibody production from B cells (see section 1.5.2), enhancing and maintaining cytolytic functions of cytotoxic CD8 T cells, regulating macrophage activation and suppressing immune responses to prevent autoimmunity (Zhu, *et al*., 2010). Furthermore, CD4 Th cells can induce cellular apoptosis via binding of the Fas ligand, a member of the TNF receptor family, to the Fas receptor present on target cells, releasing intracellular caspases. As previously described, cytokines produced by APCs and activated cells of the innate and adaptive immune system determine the type of CD4 Th effector cell, with IFN-γ and IL-12 facilitating Th1 differentiation and IL-4 for Th2 differentiation (O'Garra, 1998; Schmitt and Ueno, 2015). While Th1 cells are involved in immune responses, including macrophage activation, against intracellular bacteria and viruses and characteristically produce IL-2 and IFN-γ, Th2 secreting IL-4, IL-5 and IL-13 cells develop a response to allergens or helminth antigens and are crucial in assisting antibody production by B cells (Zhu and Paul, 2010). A cross-talk between Th1 and Th2 cells inhibit each other to maintain homeostasis. However, an imbalance between them can lead to certain diseases. Thus, a Th1 cell overload could lead to autoimmune diseases recognizing self-antigens as seen in MS, and a pre-dominant Th2 response could cause allergic reactions (Okada *et al.*, 2010). Lately, there has been proof for differentiation into other T cell subsets, including Th17 and Th9 cells via the cytokines transforming growth factor (TGF)-β, IL-6 and IL-23, and TGF-β and IL-4, respectively. Th17 cells produce IL-17 and IL-22 and are considered to be highly pathogenic, particularly when involved in CNS autoimmunity as seen in MS patients (Stockinger and Omenetti, 2017). Th9 cells produce IL-9 which has various functions, including mast cell activation, inducing Th17 proliferation as well as promoting

cytokine production from APCs (Kaplan, Hufford and Olson, 2015). They also promote regulatory T cell (Treg) function, another important Th cell subset. Tregs produce inhibitory cytokines including TGF-β and IL-10 and are involved in maintaining immunological unresponsiveness to both self- and non-self-antigens (Levine *et al.*, 2017). All the cytokines produced and released by Th cells or Tregs achieve their functions by either activating or blocking receptors or by inducing conformational changes in their corresponding receptors, which then induces or blocks intracellular signalling.

1.5.1.3 Cytotoxic CD8 T cells.

Activated CD8 T cells differentiate into cytotoxic CD8 T cells, which secrete either Th1-like or Th2-like cytokines. Both cytotoxic CD8 T cell types kill their target cells upon cell lysis (Sad, *et al*., 1995; Cox, *et al*., 2011). In addition to producing cytokines, cytotoxic CD8 T cells primarily produce cytotoxins including perforin and granzymes, which are stored in special cytoplasmic secretory lytic granules and released via exocytosis when binding to their target cell (Jenne and Tschopp, 1988; Voskoboinik, *et al*., 2015). When released, perforin either forms pores in the plasma membrane of a target cell by penetrating the lipid bilayer, resulting in necrosis or it mediates the entry of granzymes via passive diffusion, inducing apoptosis (Waterhouse *et al.*, 2006; Spicer *et al.*, 2017). Just like cytokines, cytotoxins can act locally or at a distance, and sometimes target bystander cells (Fleischer, 1986).

1.5.1.4 T cell reactivation.

Effector T cells only perform their function when they encounter their specific antigen displayed on the surface of any target cell at a site of inflammation. This happens after they have left their activation site, entered the blood stream and migrated into a specific inflamed tissue. If this would not be the case, effector T cells could induce inflammation anywhere in the body as soon as the T cell has left its activation site. In order for effector T cells to get reactivated and perform their function, antigen recognition by effector T cells does not require

a costimulatory signal, as with naïve T cell activation (Berard and Tough, 2002; MacLeod, *et al*., 2010). Although, activated T cells do express higher levels of cell-adhesion molecules than naïve cells and lose the cell surface molecule L-selectin in order to prevent recirculation through peripheral lymphoid tissues (Chao, *et al*., 1997; Venturi *et al.*, 2003). Alternatively, they express integrins, so they can bind to vascular endothelium allowing for entry into inflamed tissue sites. The exact mechanism by which effector T cells can enter inflamed tissues, including the CNS will be explained later on (see section 1.8).

1.5.1.5 T cell migration/chemotaxis.

T cell migration - or immune cells in general - plays an important role in many biological processes, including normal development, immune responses, tissue cell infiltration and wound healing. It relies on a concerted action of chemosensory stimuli, also referred to as chemokines/chemoattractants, dynamic reorganization of the (actin) cytoskeleton, formation and release of cell-matrix contacts as well as local ion homeostasis across the plasma membrane. Most migration is directed via a phenomenon known as chemotaxis, in which specific chemokine receptors induce cell movement towards a concentration gradient of corresponding chemokine ligands. These chemokines are typically classified in four subfamilies based on their structural shape, related to the number and spacing of conserved cysteine residues at the N-terminal site (C, CC, CXC and CX3C) and their corresponding receptors are comprised of a large family of seven transmembrane domain G protein-coupled receptors (Murphy *et al.*, 2000). Different chemokines therefore attract different cell types and are thus involved in various processes. Hence, chemokines including CXCL8, CCL2, CCL5, and CXCL12 are known to be released by damaged tissue in order to attract cells that promote wound healing (Ridiandries et al., 2018). Furthermore, several chemokines receptors, including CCR2, CX3CR1, CCR5, CXCR3 and CXCR2 are expressed on monocytes and microglia which play on important role in Aβ and tau pathology in Alzheimer's disease (AD) (Guedes *et al.*, 2018). In contrast to directed migration, unregulated cell migration is also

known to be a hallmark of disease, including secondary tumour formation observed in cancer, due to the migration of metastatic cells away from the primary tumour. In addition, cells can also be guided by mechanical stimuli, including guidance by stiffness gradients (durotaxis), intercellular tension (plithotaxis), intercellular force gradients (cohesotaxis) and substrate (extracellular matrix (ECM)) deformation (Roca-Cusachs *et al.*, 2013; van Helvert, Storm and Friedl, 2018).

1.5.2 B cells.

B cells are a class of lymphocytes that are involved in humoral immunity, as such, they produce and secrete antibodies following activation (Liu *et al.*, 2010). These antibodies are the secreted form of B cell receptors (BCRs) and bind pathogens or their toxic products in extracellular spaces of the body with their antigen-binding fragment (Fab) variable region and thereby either neutralize toxins, inhibit viral infections, block bacterial adherence to host cells or activate classical pathway of complement (Murphy, 2011). They perform these functions via blocking ligand-receptor interactions, causing cell lysis through activation of complement-dependent cytotoxicity (CDC), interacting with crystallisable fragment (Fc) receptors on effector cells to engage antibody-dependent cellular cytotoxicity (ADCC) or signalling phagocytes to ingest pathogens (Chan and Carter, 2010; Bournazos and Ravetch, 2017). When antibodies bind to self-antigens they are called auto-antibodies which can cause a variety of chronic autoimmune diseases, including auto-antibodies targeting NMDA receptors in schizophrenia (Masopust *et al.*, 2015).

1.5.2.1 B cell activation

Before B cells differentiate into antibody-secreting plasma cells, antigen recognition is required to trigger signalling pathways that initiate subsequent B cell activation similar to T cells. Activation of naïve B cells in peripheral lymphoid tissues is elicited by direct binding of antigens, giving an activation signal that comes either directly from antigen recognition itself

or from extensive BCR crosslinking (Shikh *et al.*, 2010). However, some antigens are not sufficient in directly activating B cells and therefore T cells help is required. In this so-called T cell-dependent B cell activation, T cells recognize the same antigen as naïve B cells and provide an accessory signal that facilitates B cell activation (Figure 1.9). Before a Th cell can interact with a naïve B cell, the antigen bound to the BCR is internalized and then re-presented on the cell surface in the form of a peptide loaded into the peptide-binding groove of MHC II molecules (Murphy, 2011). Th cells then deliver activating signals to B cells, including a costimulatory CD40/CD40L signal on B/T cells, respectively as well as IL-4 (Rush and Hodgkin, 2001) and IL-21 cytokines (Kuchen *et al.*, 2007), ensuring B cell proliferation and differentiation into antibody-producing plasma cells or memory cells (Suan, *et al*., 2017).

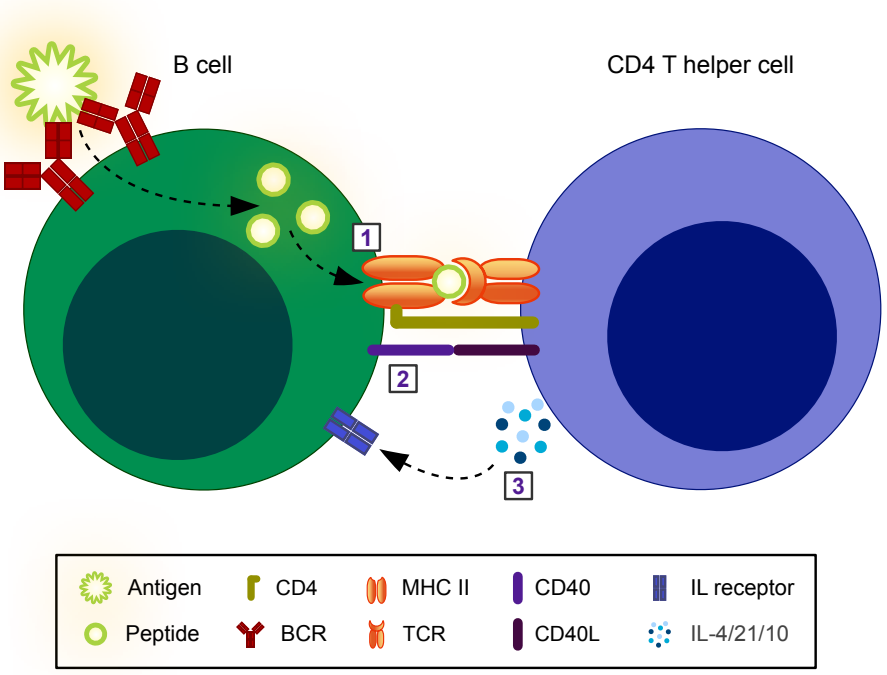

Figure 1.9. Schematic representation of T cell-dependent B cell activation. (1) Antigen is taken up by B cell receptors and degraded into short peptide fragments, to be presented to the TCR of an activated T cell. (2 & 3) The T cell then delivers activating signals via the CD40/CD40L costimulatory molecules and production of IL-4/21/10 which bind to IL-receptors on B cells.

1.5.2.2 Antibody-secreting plasma cells

When activated B cells differentiate into plasmablasts, they intensively produce low-affinity IgM isotype antibodies. After further differentiation into plasma cells by various cytokines, they will be able to generate a more effective humoral response with high-affinity IgG, IgE or IgA isotype antibodies, which are more effective in battling a chronic inflammatory state or reinfection. This is achieved by several modifications to the Ig variable and constant region genes of B cells, including somatic hypermutations in the Ig heavy and light chains enabling affinity maturation and Ig class switching enabling specific effector functions, respectively (Papavasiliou and Schatz, 2002; Stavnezer, *et al*., 2008; Tangye and Tarlinton, 2009). These DNA rearrangements are directed by different cytokines. IL-4 preferentially induces switching to IgG1, IgG4 and IgE (Lebman and Coffman, 1988; Meli *et al.*, 2017; Maehara *et al.*, 2018), while TGF-β promotes switching to IgG2b and IgA (Garcia *et al.*, 1996; Dedobbeleer *et al.*, 2017), IL-5 to IgA (Harriman *et al.*, 1988) and IFN-γ to IgG2a (Snapper and Paul, 1987). Furthermore, CD40L-CD40 interactions are highly associated with class switching, as it is shown that people who have a genetic deficiency of CD40L produce high levels of low-affinity IgM antibodies and lack the other high-affinity antibodies (Etzioni and Ochs, 2004).

1.6 The CNS: an immune-specialised site.

Whilst it is widely recognised that the CNS and immune system are cooperating with and regulating each other, the CNS has for a long time been considered an immune privileged site, completely absent of immunological surveillance. This concept was attributed by the fact that the three most important features involved in classical immune surveillance of most tissues in the human body were lacking in the CNS. These three features include APCs, MHC presentation on the surface of CNS parenchymal cells and lymphatic drainage (Louveau, *et al*., 2015; Engelhardt, *et al*., 2017). However, this immune privileged concept has been re-evaluated drastically. It has been shown that under certain circumstances astrocytes and microglia express MHC II molecules to initiate T cell-induced neuroprotective immune

responses and that neurons express MHC I molecules to initiate cytotoxic CD8 T cell-mediated clearance of dying neurons (Hayes, *et al*., 1987; Shrikant and Benveniste, 1996; Chevalier *et al.*, 2011). Furthermore, professional APCs including DCs accumulate in CSF and are constantly present in the meninges, perivascular spaces and choroid plexus to ensure immunosurveillance (Matyszak and Perry, 1996; D'Agostino *et al.*, 2012). In addition, a lymphatic vasculature was recently discovered in the dura mater of mouse, human and nonhuman primate brains (Aspelund *et al.*, 2015; Louveau *et al.*, 2015, Absinta *et al*., 2017). These vessels carry both fluid and immune cells from the CSF to the cervical lymph nodes. Not to mention, previously it was thought that the BBB's restrictive features, including a lack of intracellular fenestrations and tight junctions between brain endothelial cells, prevent immune cell entry (Reese and Karnovsky, 1967; Stamatovic, *et al.*, 2008). However, immune cells and mediators can infiltrate the brain parenchyma when restrictive barriers are broken down by CNS injury (Shechter, *et al.*, 2013). The BBB can also become more permeable as a result of subclinical infection, exposure to environmental toxins, drugs and some medications, but also stress and even healthy aging (McAllister and van de Water, 2009). This implies that the BBB is not static, but changes when the CNS needs it to change (see section 1.8, Banks and Erickson, 2010). Furthermore, it is evident that blood-borne leukocytes, including macrophages, DCs and T cells constantly patrol the healthy brain and the spinal cord and that microglia mediate surveillance and homeostasis in the CNS (Hickey, 2001; Ousman and Kubes, 2012). Together with the knowledge that the CNS and immune system share common ligands and receptors (Taub, 2008; Guyon, 2014), it is suggested that the CNS should be considered immune-specialised instead of immune-privileged, with immunosurveillance as an essential host defence mechanism.

1.7 Inflammation and T cells in CNS disorders.

With the recognition of the CNS as an immune-specialised site (Engelhardt and Ransohoff, 2005; Carson, *et al*., 2006; Malo *et al.*, 2018), pro- and anti-inflammatory events have been discovered in several CNS disorders (Stephenson *et al.*, 2018). Different human and animal studies of neurological diseases have shown that both the innate and adaptive immune system play an important role in CNS inflammation with aetiologies as diverse as infection, ischemia, neurodegeneration, autoimmunity and trauma (Yshii *et al.*, 2015; Prinz and Priller, 2017; Brown and Weinberg, 2018). These innate and adaptive immune responses evoked by both infectious and sterile inflammatory stimuli are at first a physiological defence in order to limit the extent of the disease, clear tissue damage and support tissue repair and regeneration (Jacobs and Tavitian, 2012; Marques *et al.*, 2016). On the other hand, these responses can also become detrimental if inflammation persists and becomes excessive, leading to worsening of tissue injury. Nevertheless, there is still a lot of debate whether the inflammatory events in CNS disorders are the cause of neuronal death or simply a manifestation of disease processes. This might differ between CNS disorders related to different inflammation aetiologies. Recently, several suggestions have been made to explain the link between inflammation and CNS disorders. Hence, it has been suggested that inflammation induces neurodegeneration or conversely that neurodegeneration generates inflammatory processes, implicating that both processes can cause harm to one another (Zipp and Aktas, 2006; Hosseini *et al.*, 2018; Wang *et al.*, 2018). In addition, inflammation and neurodegeneration can be involved in a vicious cycle, augmenting each other to worsen symptoms (Tsunoda and Fujinami, 2002; Amor *et al.*, 2014). It is also shown that other factors caused by a changed CNS environment during disease, including amyloid beta in case of AD, various metals or glutamate overload can induce inflammation, change immune cell phenotype from a protective to a destructive phenotype, and cause neurodegeneration (Mattson, 2004; Cicero *et al.*, 2017). Even though these propositions are not in agreement and differ per disease, they share the same key features.

Changes in local vasculature, activation of DAMPs or PAMPs and corresponding TLRs, expression of MHC, costimulatory and adhesion molecules, glial cell activation, macrophage and lymphocyte recruitment, pro- and anti-inflammatory cytokine production, free radical release and BBB alterations are all identified in various CNS diseases dealing with acute and chronic inflammation (Lucas, *et al*., 2006; Graeber, *et al*., 2011; Jacobs and Tavitian, 2012). How all these changes exactly correlate with each of the scenarios described above is not fully known and therefore more research needs to be performed to better understand the link between inflammation and CNS disorders. Although this limited understanding, the following section will briefly focus on the current knowledge of CNS diseases with different aetiologies in which immune cells, primarily T cells are actively involved, as many questions still remain regarding the good and the bad of these cells in the CNS.

1.7.1 T cells in autoimmune CNS disorders - multiple sclerosis.

MS is a complex autoimmune disease indicative by inflammation, demyelination, gliosis and degeneration of axons. A defect in the immune system, caused by the interplay of genetic or environmental risk factors including human leukocyte antigen (HLA)-DR15 mutation, Epstein-Barr virus (EBV), obesity and low vitamin D levels (Beecham *et al.*, 2013; Olsson, *et al*., 2016), targets neuronal myelin sheaths, resulting in impaired motor, sensory, autonomic, visual and cognitive functions. In specific, the CNS gets invaded by myelin-autoreactive T cells, the drivers of inflammatory responses in MS, and macrophages, both initiating pro-inflammatory cytokine secretion, toxin release and microglia activation. Furthermore, upregulated levels of inducible nitric oxide synthase (iNOS), complement and the cytokines IL-1, IL-12 and TNF-α are found in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, and MS patients (Raivich and Banati, 2004). T cells found in MS patients recognize self-antigen and have a memory phenotype, as such, autoreactive $CD4^+$ Th cells as well as activated CD8 T cells are found in MS lesioned brain tissues (Lock *et al.*, 2002). The generation of autoreactive $CD4^+$ Th1 cells is elevated in MS patients carrying the

HLA-DR15 haplotype and is shown to be mediated by memory B cells, as the depletion of memory B cells reduces autoreactive $CD4^+$ Th1 cell proliferation in vitro and within MS patients (Jelcic *et al.*, 2018). Whilst autoreactive CD4 Th cells including Th1 and Th17 cells are observed deep into MS lesions, cytotoxic CD8 T cells are predominantly found at the edges (Denic, Wootla and Rodriguez, 2013). Furthermore, Th1 cell numbers in the peripheral blood and CSF of MS patients are approximately 10-fold higher than Th17 cell numbers (Brucklacher-Waldert *et al.*, 2009). However, in an animal MS model, combining cuprizone-mediated demyelination with transfer of myelin-reactive $CD4^+$ Th cells, Th17 cells are the most prominent infiltrating cells in the CNS compared to Th1 cells (Baxi *et al.*, 2015). Interestingly, therapeutic approaches against cytokines released from Th17 cells, including IL-12 and IL-23 were ineffective in MS patients (Segal *et al.*, 2008). In addition to CD4 and CD8 T cells, Tregs are found in MS patients and have an altered, less effective phenotype (Viglietta *et al.*, 2004). It is suggested that they are inhibited in their functions due to the expression of the inhibitory molecule programmed death receptor 1 (PD-1) (Sambucci *et al.*, 2018). Therefore, most MS therapies are based on the depletion of T and B cells with a subsequent repopulation of T and B cells but with a different cell repertoire, including more Tregs. However, at the present, there is no cure for MS and the exact mechanism of how T cells contribute to disease manifestation is not yet fully understood and requires more research.

1.7.2 T cells in ischemic CNS disorders – stroke.

Cerebral vessel occlusion leading to cerebral ischemia is the major cause of a stroke, with the rupture of a diseased or abnormal vessel leading to a neurotoxic haematoma within the brain being the second most common subtype. In both ischaemic and haemorrhagic stroke, a sudden loss in sufficient oxygen and glucose supply can lead to a series of events, including cellular metabolic failure, oxidative stress, excitotoxicity, BBB damage, thrombosis and inflammation, all resulting in neuronal degeneration (Chauhan and Debette, 2016). Especially inflammatory processes are evident during the acute phase until the post-injury recovery phase. Autoimmune

disorders or complications in systemic inflammatory diseases can also manifest into a stroke (Macrez *et al.*, 2011). In addition to resident microglia, infiltrating neutrophils are the first immune responders to DAMPs in stroke (Ruhnau *et al.*, 2016), with lymphocytes and macrophages infiltrating the brain within a day. While this immune cell infiltration is critical in removing death cells and promoting regeneration, these inflammatory cells can also exacerbate the caused damage. In experimental animal stroke models induced by transient middle cerebral artery occlusion (tMCAO) as well as in stroke patients, T cell infiltration has been observed around the infarction side as early as 24 hours after injury (Schroeter *et al.*, 1994; Ortolano *et al.*, 2010; Fumagalli *et al.*, 2011). Both $CD4^+$ Th cells and cytotoxic $CD8^+$ T cells are found to contribute to inflammatory and thrombogenic responses and corresponding neurological deficits in experimental stroke (Yilmaz *et al.*, 2006). As such, Th1-derived IL-1 and TNF-α have a direct pathogenic involvement in experimentally-induced and human stroke (Wilkins and Swerdlow, 2015; Sobowale *et al.*, 2016). Indeed, blocking the IL-1R with an antagonist improves stroke outcome in young and co-morbid rats (Pradillo *et al.*, 2017). When TNF-α is administered prior to disease onset, it reduces infarct size and therefore it is suggested to also play a neuroprotective role in stroke (Hallenbeck, 2002). Whether TNF-α has a pathogenic or neuroprotective effect depends on the signalling pathways of the TNF-receptor 1 that get activated, however the mechanisms involved remain unclear. In contrast to Th1 cells, Th2 cells play a neuroprotective role in stroke pathogenesis (Arumugam *et al*., 2005). Furthermore, IL-17-producing T cells have been found to be involved in the delayed phase of cerebral ischaemia-reperfusion (I/R) when neuronal apoptosis occurs (Shichita *et al.*, 2009). Most of these cells, however accumulate in the leptomeninges instead of infiltrating the CNS parenchyma, where they control monocytes and neutrophils trafficking (Benakis *et al.*, 2016). T cell-induced detrimental effects in stroke are counteracted by $CD4^+CD25^+Foxp3^+$ Tregs, which produce the anti-inflammatory cytokine IL-10 that antagonises lymphocyte and microglia release of TNF-α and IFN-γ (Liesz *et al.*, 2009). In contrast to their neuroprotective

properties, Tregs have also been shown to promote and worsen experimental brain ischemia/reperfusion injury by inducing cerebral microvasculature dysfunction via LFA-1/ICAM-1 interactions with the ischaemic brain endothelium (Kleinschnitz *et al.*, 2013). The dual role of some mediators in stroke progression indicates that plenty questions remain regarding the role of the adaptive immune system in this complex disease.

1.7.3 T cells in neurodegenerative disorders - Alzheimer's disease.

AD is identified as a complex neurodegenerative disease associated with progressive memory loss and behavioural changes. The exact aetiology of AD is still unknown. However, it is considered a multifactorial aspect, being the result of complex interactions between genetic, environmental, gender-related, educational, lifestyle-related and contextual (e.g., head injury) risk factors (Lindsay *et al.*, 2002; Xu *et al.*, 2015). To that end, many molecular mechanisms have been proposed to explain the aetiology of AD, as such, amyloid-β (Aβ) plaques and neurofibrillary tangles (NFT), first observed by Alois Alzheimer (Alzheimer, 1907; Lansdall, 2014), are the two major pathological processes. Aβ is considered to interfere with synaptic transmission leading to neuronal cell death (Mattson, 2004; Mucke and Selkoe, 2012) and the formation of NFTs blocks the transportation of nutrients throughout neurons, disrupting neuronal cytoskeleton (Dickson, 2004; Mietelska-Porowska *et al.*, 2014). Beside these two major pathological processes, recently, oxidative stress and inflammation are also considered to play an important role in the pathogenesis of AD (Akiyama *et al.*, 2000; Agostinho, *et al*., 2010). Following several decades of genetic AD studies, dozens of loci have been identified through genome-wide association studies that are involved in inflammatory pathways in AD patients (Tosto and Reitz, 2013; Zhang *et al.*, 2015; Broce *et al.*, 2018). As such, it has been shown that resident microglia and their upregulation of cytokines and chemokines are major contributors of inflammation in AD. Hence, surrounding microglial cells can be activated by oligomeric Aβ which then secrete pro-inflammatory cytokines, including TNF-α and IL-1β (Rogers and Lue, 2001). Once activated, these microglia recruit astrocytes and thereby

enhance the inflammatory process. Furthermore, it is proven that Aβ upregulates chemokine and ROS production (Perry and Castellani, 1998; Xia and Hyman, 1999; Wang *et al.*, 2010) and activates the complement system (Yasojima *et al.*, 1999; Lashkari *et al.*, 2018). All these factors contribute to neuronal dysfunction and cell death, either in a solitary matter or concertedly. In contrast to a pro-inflammatory response in AD, neuronal dysfunction and cell death itself can provoke an anti-inflammatory response in which microglia migrate to neuronal debris and promote phagocytosis in order to prevent glutamate-induced neurotoxicity (Mizuno, 2012). In addition to microglia, an increased number of activated T cells have been found in the CNS of AD patients (Togo *et al.*, 2002) as well as in their CSF (Lueg *et al.*, 2015). As such, cytotoxic CD8 T cells have been observed in the brains of AD patients, which was significantly correlated with tau pathology but not Aβ plaques (Merlini *et al.*, 2018). Furthermore, when AD mice with a mutation in the presenilin (PS) and amyloid precursor protein (APP) genes involved in Aβ overproduction are crossed with a mouse deficient in T and B cells, Aβ levels were reduced in the brain, but cognition was not improved, suggesting that T and B cells contribute to AD pathology (Späni *et al.*, 2015). Th17 cells in specific have also been observed in the brains of an $A\beta_{1\text{-}42}$-induced AD rat model and contribute to neurodegeneration via the release of proinflammatory cytokines as well as the contact-dependent interaction with Fas on neurons (Zhang *et al.*, 2013). In contrast to a pathogenic role of the adaptive immune system in AD, 5x familial AD (FAD) mice who were crossed with T, B and NK cell-deficient mice showed a more severe Aβ pathology, suggesting a neuroprotective role of the adaptive immune system (Marsh *et al.*, 2016). Furthermore, Tregs have been found in mild cognitive impaired AD patients, however these Tregs have a unique phenotype, PD-1 Tregs, which acquire the greatest immunosuppressive function and therefore could conversely contribute to AD pathogenesis (Saresella *et al.*, 2010). Hence, it has been shown that depleting of $Foxp3^+$ Tregs in the 5x FAD model reduces amyloid plaque load, mitigates neuroinflammatory responses and enhances cognition (Baruch *et al.*, 2016). With

the dual role of various T cells in AD pathology, the exact role of T cells remains unknown and requires further investigation.

1.8 T cell entry into the inflamed and non-inflamed CNS.

Having acknowledge that the immune system constantly interacts with the CNS and that immune cells, particularly T cells patrol and migrate into the CNS in physiological and pathophysiological conditions, it has become evident that T cells play a major role in inflammatory CNS disorders. Therefore, the focus of this study will be on T cells. These T cells can be initiators and mediators of disease as in MS, or they can contribute to disease progression as seen in AD and stroke. What their exact function and mechanism in CNS disorders is, is not fully understood, however it is fairly established how T cells migrate into the CNS. As in any other tissue, circulating immune cell recruitment into the CNS is mediated by a phenomenon called leukocyte extravasation (Ley *et al.*, 2007; Engelhardt and Ransohoff, 2012). It utilises the interaction between adhesion and/or signalling molecules on immune cells and endothelial cells lining the vessel wall. In brief, leukocytes first slowdown in the bloodstream near the site of inflammation or tissue damage, due to dilated vessels and a consequent slower blood flow. As the leukocyte rolls along the blood vessel wall, cell adhesion molecules (CAMs) called selectins, including L- (on lymphocytes), E- (on endothelial cells) and P-selectin (on platelets and endothelial cells) bind to their corresponding carbohydrate ligands loaded onto proteins presented by leukocytes (Zarbock *et al.*, 2011). Arrest chemokines, including the chemokine ligand (CCL)-19, CCL21, or CXCL12, attached to proteoglycans on the surface of endothelial cells then bind to specific chemokine receptors on leukocytes, which subsequently signal the cell to trigger a conformational change and activate integrins on the surface of the rolling leukocyte (Montresor *et al.*, 2012). These activated integrins include the α_4 integrins (on monocytes, eosinophils and various B and T cells), very late antigen (VLA)-4 and $\alpha_4\beta_7$, and the β_2 integrins, LFA-1 (on all leukocytes) and macrophage-1 antigen (Mac-1, on neutrophils and monocytes), and bind to their corresponding

endothelial CAMs, resulting in a final firm adhesion, arrest and flattening of leukocytes (Nourshargh, *et al.*, 2016). This initiates the next step, also known as 'diapedesis' in which the cytoskeleton is reorganized and the basement membrane's extracellular matrix proteins are broken down by matrix metalloproteinase (MMP) enzymes, allowing leukocytes to squeeze between the endothelial cells and enter the tissue (Vestweber, 2015). Furthermore, leukocytes can extravasate through either tight junctions (paracellular migration) or endothelial vesicles (transcellular migration) (Martinelli et al., 2014).

1.8.1 Leukocyte infiltration routes into the CNS.

Peripheral leukocyte infiltration into the CNS parenchyma takes place via three distinct routes including a vascular route, a blood-CSF route and a meningeal lymphatic route (Figure 1.10, Engelhardt and Ransohoff, 2012; Prinz and Priller, 2017). Following the vascular route, leukocytes migrate from the blood to the parenchymal perivascular space crossing the BBB located at the brain parenchymal microvessels into the CNS parenchyma. In specific, leukocytes from the internal carotids enter the CNS parenchyma by passing through the post-capillary venules and eventually extravasate at the post capillary venules. Leukocytes migrate thus into the perivascular space, where they form perivascular cuffs and stimulate leukocyte activation. This prepares leukocytes for a second and final migration into the parenchyma, where they encounter glia limitans, also called astrocyte borders. In steady state conditions, glia limitans restrict leukocyte entry as they facilitate the tightness of the BBB by secreting soluble factors that maintain the tight junctions between endothelial cells (Sofroniew, 2015; Lécuyer, Kebir and Prat, 2016). However, at sites of tissue damage or tissue inflammation, astrocytes form glial scars, composed of newly proliferated, elongated astrocytes, lining between viable CNS neuronal parenchyma and non-neuronal cells (Wanner *et al.*, 2013).

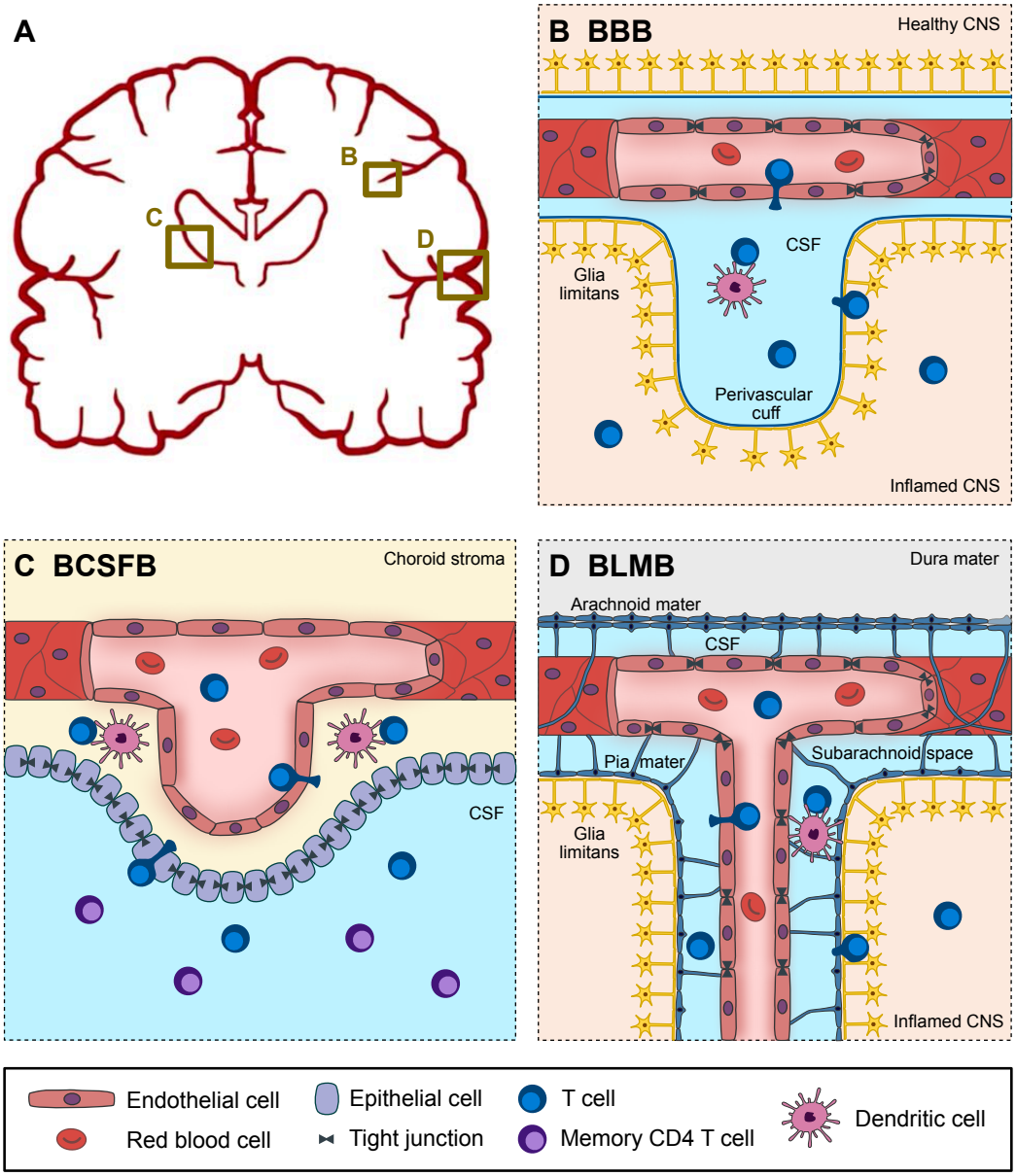

Figure 1.10. Schematic representation of T cell infiltration routes in the CNS. (A) Schematic representation of the brain illustrating the three different locations for T cell infiltration. T cells can infiltrate the CNS via (B) a vascular route at the BBB, (C) a blood-CSF route at the choroid plexus and (D) a meningeal lymphatic route in the subarachnoid space.

During inflammatory conditions, astrocytes either exert pro-inflammatory effects by releasing molecules that disrupt the BBB and therefore promote extravasation, or anti-inflammatory effects which promote BBB repair and subsequently restrict leukocyte entry (Sofroniew, 2009). Following the blood-CSF route, leukocytes traverse from the blood to the CSF crossing the choroid plexuses located in the third and fourth ventricles, also known as the blood-CSF-barrier (BCSFB) ((Schwartz and Baruch, 2014). In this scenario, leukocytes firstly extravasate the fenestrated endothelium of the choroid plexus stroma, then travel through the stroma to eventually migrate right into the CSF via the epithelial cells of the choroid plexus. Following the meningeal lymphatic route, leukocytes travel from the blood to the subarachnoid space crossing the blood-leptomeningeal-barrier (BLMB), where they migrate from the internal carotid artery into the subarachnoid space and the Virchow-Robin perivascular space via post capillary venules at the pial surface (Weller *et al.*, 2018).

1.8.2 T cell infiltration/migration in the CNS.

In the healthy CNS, mostly activated lymphocytes, including central and effector memory T cells, are detected specifically in CSF containing regions. Hence, $CD4^{+}CD45RA^{-}CD27^{+}CD69^{+}$-activated central memory T cells are recruited into the healthy human CSF through the interactions of T cell-derived L-selectin with its corresponding ligand and the C-C chemokine receptor type 7 (CCR7) on T cells, which can bind to CCL19 expressing endothelial cells of the choroid plexus (Kivisäkk *et al.*, 2003). It is therefore suggested that under non-inflamed conditions, only activated T cells can breach the protective barriers into the CNS, as T cells only express selectins and integrins required for leukocyte extravasation in an activated state. In agreement, whilst naïve T cells express CCR7, they show stationary behaviour in healthy, non-inflamed CNS parenchyma as they cannot find sufficient migratory signals (Herz *et al.*, 2011). However, while activated T cells enter the CSF under healthy conditions, these T cells do not invade the CNS parenchyma (Ransohoff and Engelhardt, 2012). At the healthy BBB, lymphocyte extravasation is not as effective as during

inflammatory conditions and only few (activated) cells are detected in perivascular locations (Hickey, 1991). This is probably due to a lack of selectins expressed at the BBB's endothelial cells, resulting in a lack of rolling and arrest during T cell extravasation (Kivisäkk *et al.*, 2009). Furthermore, immunological mechanisms including abluminal endothelial expression of CXC-chemokine ligand 12 (CXCL12) and IL-25 prevent leukocyte invasion and maintain barrier sealing (McCandless *et al.*, 2006; Sonobe *et al.*, 2009). Therefore, the BBB is often considered a true barrier during non-inflammatory conditions, while the BCSFB is recognised as an immunosurveilance site (Shechter, *et al*., 2013), as T cell entry efficiency is much higher at this border (Carrithers *et al.*, 2000). Furthermore, CSF contains anti-inflammatory properties skewing infiltrated T cells to an alternatively activated or regulatory phenotype (Gordon *et al.*, 1998; Mueller *et al.*, 2009). A negligible T cell extravasation at the meningeal crossroads under normal conditions suggest that also the BLMB is a true barrier that does not have immunoregulatory properties (Shechter *et al*., 2013). Breaching of both true barriers as well as the BCSFB in various pathologies, however does facilitate T cell infiltration into the CNS parenchyma. Hence, both naïve and activated T cells are found within the inflamed CNS at all three locations (Cose *et al.*, 2006; Bartholomäus *et al.*, 2009; Herz *et al.*, 2011; Engelhardt and Ransohoff, 2012). Whilst naïve T cells can enter the inflamed CNS, they do require antigenic stimulation for activation (see section 1.6, Krakowski and Owens, 2000). Furthermore, in the inflamed CNS, stromal cells in the perivascular space generate CCR7 ligands, including CCL19 and CCL21, which support recruitment and local reactivation of cytotoxic $CD8^+$ T cells that control protective immunity in the CNS (Cupovic *et al.*, 2016).

1.9 T cell reactivation in the CNS.

Following T cells activation in peripheral lymphoid organs, T cells infiltrate the CNS and can initiate responses against neurotrophic pathogens, CNS auto-antigens and damaged or dying neurons. However, effector T cells can only perform their function when they are reactivated at the site of inflammation. Hence, peripherally activated T cells in the CNS patrol their

surroundings for their target pMHC presented by APCs or neurons in order to become reactivated and initiate effector responses. These effector responses include soluble mediator production in the form of cytokines, which induces cell death or promotes neuroprotection, but also the recruitment and activation of other inflammatory cells, controlling their functions. APCs involved in reactivation processes in the CNS are professional APCs, including DCs, macrophages, endothelial cells, microglia, as well as non-professional astrocytic APCs. Furthermore, the degree of inflammation in the CNS initiated by T cell reactivation is dependent on the reactivation strength (Kawakami *et al.*, 2004). In specific, the affinity between the TCR and its associated pMHC ligand, as well as the number of pMHC complexes on the surface of APCs determines the magnitude and type of response generated (Goverman, 2009). The ability of an activated T cell to enter the brain is not linked with the degree of inflammation, supported by the fact that T cells leave the brain/CSF into cervical lymph nodes via the cribroid plate and nasal mucosa (Goldmann *et al.*, 2006) once they do not find their target pMHC and thus not get reactivated within 24 hours (Hickey, 1991, Hickey, 1999).

1.9.1 Microglia-induced T cell reactivation.

While microglia are the largest population of phagocytic cells in the CNS, they are not considered as typical APCs as there is currently no proof that they migrate to draining lymph nodes, once taken up antigen, to activate naïve T and B cells. Thus, it is suggested that microglia wait in the CNS to reactivate infiltrating T cells (Carson, 2002; Schetters *et al.*, 2018). Indeed, microglia constitutively express very low levels of MHC II molecules in homeostatic conditions required for antigen presentation to reactivate T cells. These MHC II molecules are upregulated in response to a wide variety of activating signals, including infection, aging and IFN-γ, (Olson, Girvin and Miller, 2001; VanGuilder *et al.*, 2011; Smith *et al.*, 2013). This constitutive and inducible MHC II expression is dependent on the class II transactivator (CIITA), a transcriptional coactivator controlled by the alternative usage of three different promotors. Hence, while constitutive MHC II expression is under the control of type

I and type III promotors, similar to DCs and B cells, respectively, IFN-γ-induced MHC II expression is controlled by the type IV promotor, as observed in a variety of MHC II negative cell types (Harton and Ting, 2000; Pai *et al.*, 2002). All three types of promotors are found in microglia (O'Keefe *et al.*, 2001). Furthermore, MHC II (HLA-DR in humans) expression is highly upregulated on microglia in AD, PD and MS (Walker and Lue, 2015; Hendrickx *et al.*, 2017). Not much is known though about the expression of MHC I on microglia, however it has been shown that neonatal and adult mouse microglia can cross-present exogenous antigens via their MHC I molecules (Beauvillain *et al.*, 2008). Whilst microglia express MHC II molecules, in order to present antigen via these molecules, microglia also require the ability to process antigen. Interestingly, while nonactivated microglia cannot process antigen efficiently, IFN-γ or viral infection-induced microglial activation does result in efficient endogenous viral antigen- and exogenous myelin antigen-processing and presentation (Olson, *et al*., 2001). Given that nonactivated microglia cannot process antigen and that empty MHC II complexes are quickly degraded before they reach the plasma membrane, both phenomena could explain the low to undetectable MHC expression on nonactivated microglia. Furthermore, while co-stimulatory signals are not necessary for effector or memory T cell reactivation, activated microglia can express the costimulatory B7-1 and B7-2 molecules in addition to CD40, ICAM-1 and vascular adhesion molecule (VCAM)-1 (Peterson *et al.*, 2002; Chen *et al.*, 2006; Miklossy *et al.*, 2006; Wlodarczyk *et al.*, 2014). Considering all this, it is suggested that microglia are professional APCs and can (re)activate both naïve T cells and activated T cells found in the diseased CNS. Hence, microglia were shown to efficiently reactivate both Th1 and Th2 cells via MHC II molecules presenting the ovalbumin$_{323-339}$ peptide and the costimulatory molecule CD40 and adhesion molecule ICAM-1 (Aloisi *et al.*, 1998). However, microglia are not as effective in antigen presentation compared to perivascular-, meningeal- and choroid plexus macrophages (Nayak *et al.*, 2012, Wlodarczyk *et al*., 2014). In addition,

the most important professional APC is considered the perivascular macrophages, as T cells encounter these macrophages at first when entering the CNS.

1.9.2 Astrocyte-induced T cell reactivation.

Astrocytes have been shown immunocompetent cells, regulating innate and adaptive immune responses and therefore considered to play a crucial role in CNS immune function (Colombo and Farina, 2016; Almad and Maragakis, 2018). Indeed, dependent on their activation state, astrocytes may induce inflammatory responses leading to either tissue damage or promote immunosuppression, resulting in tissue repair. They can also reactivate infiltrating, previously activated T cells in the CNS via MHC II molecules present on the cell surface (Constantinescu *et al.*, 2005). However, MHC II expression on astrocytes requires CIITA type IV promotor activation (Dong, *et al*., 1999) and thus MHC II expression is only upregulated following IFN-γ-induced astrocytic activation. As MHC II expression is not constitutively upregulated, astrocytes are considered non-professional APCs. In contrast to microglia, astrocytic MHC II induction can be silenced by cortical neurons, via a mechanism that is not fully understood yet (Tontsch and Rott, 1993). Furthermore, astrocytes can upregulate B7-1 and B7-2 surface expression following IFN-γ stimulation (Nikcevich *et al.*, 1997; Zeinstra *et al.*, 2006). However, B7-1 expression can only be upregulated in mature astrocytes (Soos *et al.*, 1999). In addition, human immunodeficiency virus (HIV)-1 transactivator of transcription (Tat) protein has been shown to significantly increase the adhesion molecules ICAM-1 and VCAM-1 on astrocytes (Song *et al.*, 2007). Thus, astrocytes are considered to (re)activate both naïve and activated T cells, but only in an inflammatory environment. Indeed, astrocytes induce naïve CD4 Th cell and cytotoxic CD8 T cell proliferation in the presence of IFN-γ (Cornet *et al.*, 2000), suggesting that astrocytes also upregulate MHC I molecules. Hence, MHC I-positive astrocytes are present in the lesion edge, gliotic lesion centre and in white matter of human MS brain tissues (Ransohoff and Estes, 1991). While astrocytes can present myelin oligodendrocyte glycoprotein (MOG, autoantigen in MS) peptides to MOG-specific T cells,

inducing a mixed Th1/Th2 response, they are poor at processing and subsequent presentation of native MOG protein (Kort *et al.*, 2006). This observation was consistent with the poor processing of ovalbumin protein into ovalbumin epitopes and questions the ability of astrocytes to act as APCs (Aloisi *et al.*, 1998). However, astrocytes have been shown to efficiently process and present proteolipid protein (PLP, autoantigen in MS) and myelin basic protein (MBP, autoantigen in MS) proteins into peptides and suggest that astrocytes use different forms or antigen presentation (Soos *et al*., 1998). Furthermore, whilst astrocytes are considered APCs in inflammatory CNS diseases and mainly reactivate Th2 cells, microglia are more efficient than astrocytes in activating naïve CD4 T cells and reactivating Th1 cells (Aloisi *et al*., 1998). It could therefore be implied that astrocytes counteract the potentially pathogenic Th1 induced responses by reactivating Th2 cells, however this still requires more research.

1.10 Neuronal-T cell interactions in the CNS.

T cell migration towards the injured CNS and its reactivation within the CNS by professional and non-professional APCs is becoming better defined. However, many questions remain regarding the subsequent beneficial or detrimental nature of the adaptive immune system in the CNS. Whilst it was first thought that the adaptive immune response to injury was largely detrimental, nowadays CNS rodent disease models lacking T and B cells worsen disease, indicating a beneficial role (Wraith and Nicholson, 2012; Kipnis, 2016; Klein and Hunter, 2017). Thus, what drives a T cell to cause immunopathological damage in certain anatomical sites of the CNS or otherwise dampen inflammation and re-establish tissue homeostasis is not fully understood. It is known though that T cell-mediated cytokine release following APC-induced reactivation in the CNS is one mechanism involved in neuroinflammation (see section 1.10.2). In contrast, T cells can also exert their functions via direct neuronal antigen or antigen-independent stimulation through the formation of an immunological synapse (see section 1.10.1). Therefore, it is suggested that T cells facilitate neuroinflammation in a contact-

dependent or -independent manner. However, the exact cellular and molecular mechanisms underlying both types of T cell-induced neuroinflammation are not fully understood. Furthermore, it is unknown how these interactions may be regulated or even simultaneously cross-regulate final T cell function and neuronal viability.

1.10.1 Contact-dependent neuronal-T cell interactions.

Contact-dependent neuronal-T cell interactions in the CNS can be mediated via MHC- and antigen-dependent or -independent mechanisms (Figure 1.11). In particular cytotoxic $CD8^+$ T cells interact with neurons via MHC I molecules. MHC I expression has been observed in many rodent CNS neurons (Lindå *et al.*, 1998; Needleman *et al.*, 2010; J. Liu *et al.*, 2013) as well as in human catecholaminergic substantia nigra and locus coeruleus neurons (Cebrián *et al.*, 2014). Whilst it is well-established that developing neurons express MHC I and that this molecule has various physiological roles in brain development, synaptic plasticity and axonal regeneration (Joseph *et al.*, 2011; Bilousova *et al.*, 2012; Elmer *et al.*, 2013) the precise immunological function of MHC I expression on neurons has been debated for a long time. As it was shown that MHC I surface expression is only present on electrically silent rat hippocampal cultures following IFN-γ stimulation, it was suggested that cytotoxic $CD8^+$ T cells only interact with functionally impaired or dying neurons to mediate removal of these cells (Neumann *et al.*, 1995). To further investigate whether this is true, microfluidic platforms can be used in which changes in neuronal activity can be measured in combination with lymphocyte migration behaviour (see section 1.12). Nowadays, it is known that MHC I molecules play a pivotal role in viral-mediated neuroinflammation. Hence, brain-isolated $CD8^+$ T cells exhibit a reduced motility to form a stable immunological synapse with Borna disease virus (BDV)-infected rat cortical neurons in an antigen- and MHC I-dependent manner (Chevalier *et al.*, 2011). Whilst this contact-dependent interaction increases electrical properties of rat cultured cortical neurons in the first 3 hours, neuronal apoptosis is observed in these neurons following longer incubation periods.

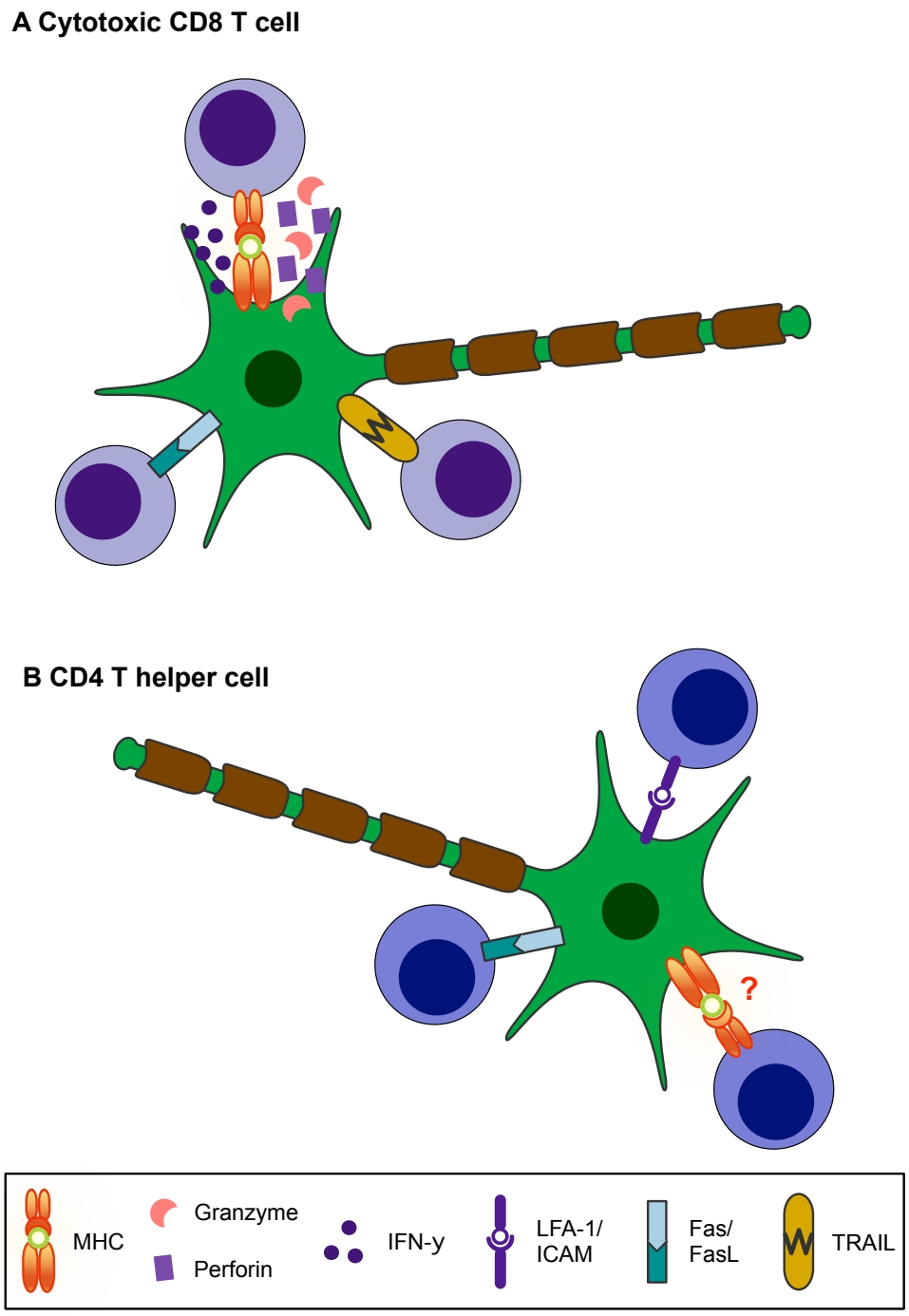

Figure 1.11. Schematic representation of MHC-dependent and -independent contact-dependent interactions between neurons and T cells in the CNS parenchyma. (A) CD8 T cells interact with neurons via their TCR:pMHC I interaction, as well as the Fas/FasL and TRAIL complexes. (B) As it is not known whether neurons express MHC II molecules, it is suggested that CD4 T cells interact with neurons via LFA-1/ICAM and Fas/FasL complexes.

Furthermore, deep cerebellar nuclei (DCN) neurons of lymphocytic choriomeningitis virus (LCMV)-challenged mice showed synapse loss and motor impairment following MHC I-dependent interaction with cytotoxic $CD8^+$ T cells releasing IFN-γ, through Janus kinase (JAK)/signal transducer and activator of transcription (STAT)-1 signalling (Kreutzfeldt *et al.*, 2013; Di Liberto *et al.*, 2018). In contrast to this pathogenic role, cytotoxic $CD8^+$ T cells can inactivate herpes simplex virus type 1 (HSV-1) by the release of lytic granules and to a lesser extent IFN-γ in HSV-1-infected mice and HSV-1-infected fibroblast, without inducing neuronal cell death (Knickelbein *et al.*, 2008). These lytic granules contain granzyme B that directly cleaves ICP4, a viral transcription factor, and thereby prevents HSV-1 reactivation. The pore forming cytolytic protein perforin is also found in lytic granules and has been shown to eliminate Toxoplasma *gondii* cysts from neurons in the brains of chronically infected mice following protective cytotoxic $CD8^+$ T cell interactions with neurons (Suzuki *et al.*, 2010). Furthermore, cytotoxic $CD8^+$ T cell-derived secretory granule proteins are capable of inducing a biphasic $[Ca^{2+}]_i$ rise in cerebellar granule neurons (CGNs), with a role for NMDA receptors (NMDARs) in the second calcium wave (Malipiero *et al.*, 1999). Perforin is also known to induce pathogenic cytotoxic $CD8^+$ T cell MHC I-mediated interactions with hippocampal cultured neurons. As such, cytotoxic $CD8^+$ T cells impair neuronal electrical signalling, resulting from perforin-mediated neuronal membrane capacitance disruption (Meuth *et al.*, 2009). This was paralleled by an increase in intracellular Ca^{2+} levels, which indicates that the interaction precedes neuronal cell death, but is not causally linked. In addition to MHC I-dependent cytotoxic $CD8^+$ T cell cytolytic mechanisms to clear West Nile Virus (WNV) infection, Fas/FasL interactions between cytotoxic $CD8^+$ T cells and neurons have been shown to limit infection in the CNS of WNV-infected mice (Shrestha and Diamond, 2007). Furthermore, cytotoxic $CD8^+$ T cells require TNF-related apoptosis-inducing ligand (TRAIL) surface expression to limit infection in the brain and spinal cord of WNV-infected mice as well as WNV-infected primary cortical neuronal cultures (Shrestha *et al.*, 2012). This is

dependent on TRAIL binding to the death receptors (DR) DR4 and DR5, which induces caspase-8-dependent apoptosis.

Whilst MHC-II molecules are present and functional in human embryonic/foetal brain-derived neural stem cells (hNSCs) and regulated independently of IFN-γ stimulation (Vagaska *et al.*, 2016), it is still unclear whether $CD4^+$ Th cells interact with neurons in an MHC-dependent manner, as there is no further significant proof for MHC II expression on neurons. It is shown though that $CD4^+$ Th cells mediate dopaminergic-induced neuronal toxicity in a PD and AD mouse model via FasL/Fas contact-dependent interactions (Brochard *et al.*, 2009; Zhang *et al.*, 2013). Furthermore, PLP-stimulated T cells induce neurotoxicity in organotypic entorhinal-hippocampal slice cultures (OSCs) via lymphocyte bound TRAIL (Aktas *et al.*, 2005). PLP- as well as ovalbumin-specific Th1 cells have also been shown to induce lethal increases in neuronal $[Ca^{2+}]_i$ levels in acute brain slices, with a role for perforin and glutamate receptors (Nitsch *et al.*, 2004). Indeed, $CD4^+$ T cells with cytotoxic activity, including the release of granzyme B and perforin have been observed in various immune responses in an MHC II-dependent manner (Takeuchi and Saito, 2017). However, the detrimental effects of Th1 cells on living brain tissue were MHC II-independent (Nitsch *et al*., 2004) and therefore it is unclear how this contact-dependent interaction mediates neuronal cell death. It is suggested though that functional immunological synapse formation can be established via the adhesion complex LFA-1/ICAM-1. In agreement with this, contact-dependent interactions between MOG-specific Th17 cells and neurons in demyelinating lesions of EAE mice result in axonal pathology, which is dependent on the LFA-1/ICAM-1 complex and independent of MHC II expression and antigen-specificity (Siffrin *et al.*, 2010). In contrast to pathogenic neuronal-$CD4^+$ Th cell interactions, encephalitogenic ovalbumin-specific T cells upregulate mouse cerebellar neuronal B7.1, B7.2, ICAM-1 and TGF-β following contact-dependent interactions, which then upregulates the transcription factor FoxP3 on these ovalbumin-specific T cells, resulting in the conversion of encephalitogenic T cells into Tregs (Liu *et al.*, 2006). These

Tregs inhibit EAE pathology, with a role for cytotoxic T lymphocyte antigen (CTLA)-4. Furthermore, $CD4^+$ Th cell-mediated enhanced neurite outgrowth was observed when mouse cortical cultures were exposed to Th1 cells, but not to Th2 or naïve T cells, which was dependent on semaphorin (SEMA)-4A (Ishii *et al.*, 2010).

Whether naïve T cells form immunological synapses with neurons is not investigated so far to the best of my knowledge. Several studies have shown that naïve T cells do not induce neurotoxicity (Giuliani *et al.*, 2003; Nitsch *et al.*, 2004). In agreement, a study in our lab showed that naïve T cells were neuroprotective against KA-induced excitotoxicity and oxygen glucose deprived (OGD)-induced cell death (Shrestha *et al.*, 2014). However, naïve T cells were not able to enhance neurite outgrowth when co-cultured with cortical neurons (Ishii *et al*., 2010). Therefore, further research is required to unravel whether naïve T cells play a protective role in the CNS and whether this is in a contact-dependent or -independent manner.

1.10.2 Contact-independent neuronal-T cell interactions.

In contrast to contact-dependent interactions between neurons and T cells in the CNS, neuroprotection and neuronal damage can be mediated via inflammatory mediators (Figure 1.12). Hence, it was shown that antigen-stimulated MBP-specific Th2 cells mediate more effective neuroprotection than Th1 and ovalbumin-specific Th2 and Th1 cells in a contact-independent manner (Wolf *et al.*, 2002). It was not further investigated which inflammatory mediator was responsible for the observed neuroprotection, but is suggested to be mediated via anti-inflammatory cytokines, including IL-10, IL-4 or neurotrophic factors, including brain-derived neurotrophic factor (BDNF) or neurotrophin (NT)-3. Whilst BDNF is mainly produced by neurons in the CNS, human MBP- or MOG-specific Th1 and Th2 cells release BDNF following antigen stimulation, which supports survival of chicken sensory neurons from the nodose ganglia *in vitro* (Kerschensteiner *et al.*, 1999). Therefore, an effective role of BDNF for the treatment of MS patients has been suggested, especially since peripheral BDNF levels in MS patients are significantly lower than healthy individuals (Văcăraş, *et al*., 2017).

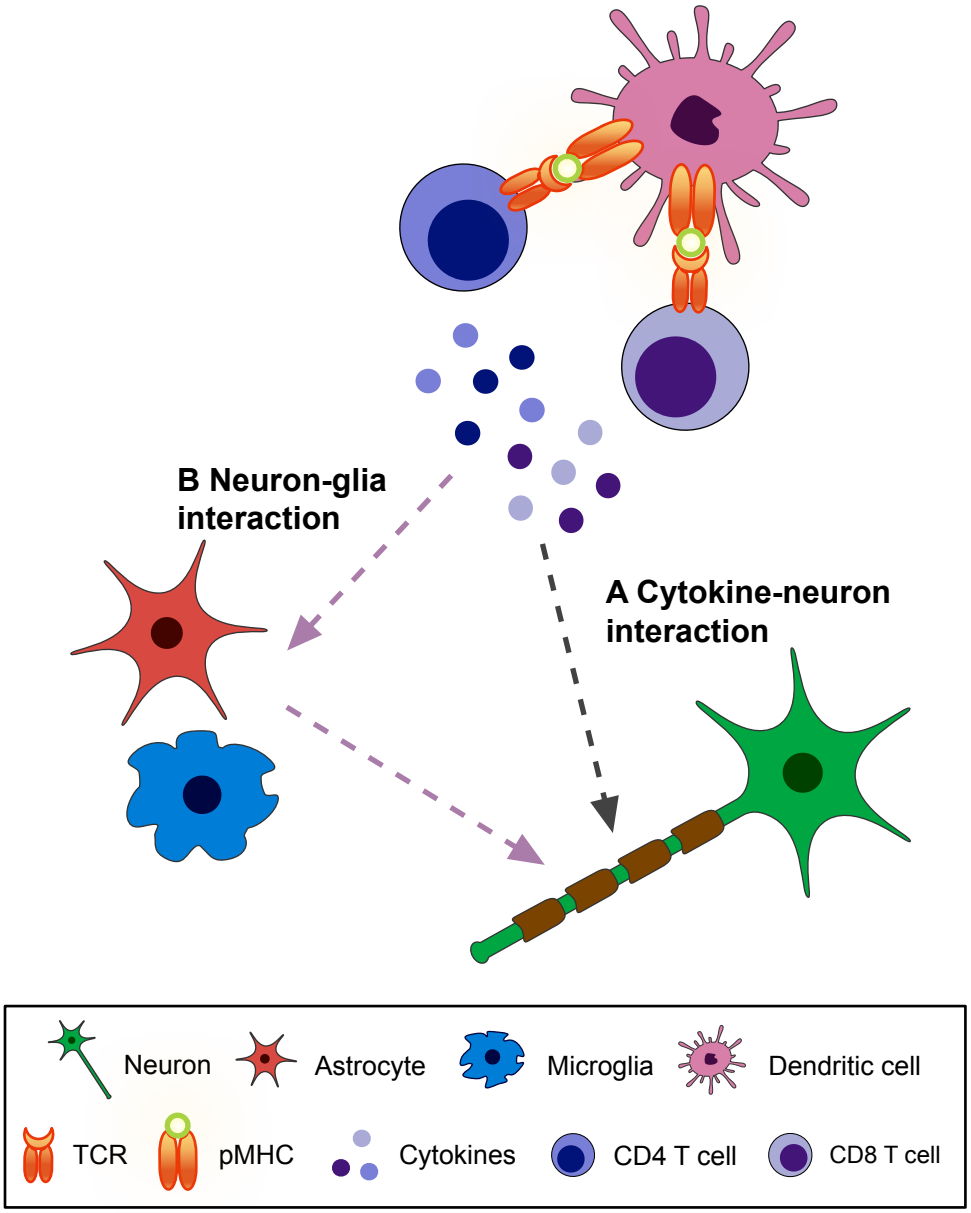

Figure 1.12. Schematic representation of contact-independent interactions between neurons and T cells in the CNS parenchyma. (A) CD4 and CD8 T cells can get reactivated in the CNS by APCs through the binding of TCRs on either CD4 or CD8 T cells and their corresponding pMHC II or pMHC I complex on the surface of APCs, respectively. These CD4 and CD8 T cells then produce cytokines which interact with neurons. (B) These cytokines can also first activate microglia and astrocytes, which then interact with neurons.

The anti-inflammatory cytokine IL-10 reduces glutamate-induced apoptotic cell numbers in rat CGN cultures by blocking caspase-3 and (nuclear factor kappa-light-chain-enhancer of activated B cells) NF-κB activity (Bachis *et al.*, 2001). IL-10-exposed rat primary cortical neurons also results in increased neuronal survival following OGD and glutamate-induced excitotoxicity, through the IL-10R and (phosphoinositide 3-kinase) PI3K/AKT and STAT-3 signalling pathways (Sharma *et al.*, 2011). Furthermore, IL-10 abolishes spontaneous $[Ca^{2+}]_i$ increases induced via repeated NMDAR activation with brief hypoxic episodes in rat cultured hippocampal neurons through the inhibition of inositol trisphosphate ($InsP_3$)-sensitive internal stores (Turovskaya *et al.*, 2012). In addition to IL-10, Th2 cell-derived IL-4 enhances neuronal survival via neuronal IL-4R activation, which induces neurotrophin signalling via AKT and (mitogen-activated protein kinase) MAPK pathways in murine optic nerve crush injury and spinal cord contusive injury models (Walsh *et al.*, 2015). Furthermore, clinical signs and axonal pathology in different chronic mouse EAE models was ameliorated by both intrathecal and intranasal IL-4 application (Vogelaar *et al.*, 2018). This study also showed that IL-4 protects against NMDA-induced cytotoxicity in dissociated cortical neurons and induces axonal growth in the cortex of WT mice, indicating that IL-4 induces regenerative plasticity. In contrast to its neuroprotective role, Th2 cell-derived IL-4 activates dorsal root ganglion (DRG) sensory neurons via the IL-4Rα and JAK signalling, causing chronic itch (Oetjen *et al.*, 2017). Other cytokines, including IL-6 and IL-17A have also been shown to be involved in neuronal excitability. Hence, chronic IL-6 exposure of primary cerebellar Purkinje neurons (CPNs) results in a reduction in AP generation and an enhanced electrical response to AMPA via the IL-6R and its intracellular signalling molecule gp130 (Nelson, Ur and Gruol, 2002). However, lowering the dose by half did not affect electrophysiological properties, but did result in increased $[Ca^{2+}]_i$ signals in response to AMPA. Furthermore, chronic exposure of rat CPNs to IL-6 results in elevated resting $[Ca^{2+}]_i$ levels, as well as dihydroxyphenylglycine (DHPG)- and K^+ chloride (KCl)-induced increases in $[Ca^{2+}]_i$ levels (Nelson, *et al.*, 2004). In contrast to the enhanced $[Ca^{2+}]_i$ release from internal stores, IL-6 exposed CGNs results in a decreased

NMDA-induced extracellular Ca^{2+} influx via InsP3 receptors and JAK/calcineurin (CaN) signalling (Z. Liu *et al.*, 2013; Ma *et al.*, 2015), suggesting that IL-6 can alter Ca^{2+} signalling via different pathways. Furthermore, IL-17A injection into the rat knee joint induces long-lasting nociceptive C fibre sensitization in response to mechanical stimuli (Richter *et al.*, 2012). This cytokine rapidly induces protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) phosphorylation in rat DRG cultured neurons and enhances neuronal excitability in the form of increased AP numbers and a decreased current needed for AP elicitation (Richter *et al*., 2012). It has also been shown that IFNs interact with neurons in a contact-independent manner. As such, IFN-α inhibits glutamate-induced EPSCs in *Cornu Ammonis* (CA)-1 hippocampal neurons and prevents high-frequency tetanic stimulation-induced long-term potentiation (LTP) via inhibiting tyrosine kinase activity and non-NMDA glutamate receptors (Mendoza-Fernández, *et al*., 2000). In addition, $CD4^+$ T cell-derived IFN-γ protects hippocampal neurons from glutamate-induced excitotoxicity, which was associated with an enhanced recovery of glutamate-induced $[Ca^{2+}]_i$ increases (Lee *et al.*, 2006). In contrast, cytotoxic $CD8^+$ T cell-derived IFN-γ has also been shown to enhance glutamate-induced neurotoxicity in mouse primary cortical neurons via the AMPA receptor subunit GluR1 (GluA1, Mizuno *et al.*, 2008). In specific, GluA1 is phosphorylated at the serine 845 position via the JAK1-2/STAT1 pathway, which then induces increases in $[Ca^{2+}]_i$ levels, followed by NO production and dendritic bead formation. Furthermore, IFN-β1a, used as treatment for MS, reduces striatal EPSC amplitude via the NMDA receptor subunit (GluN2a), requiring Ca^{2+} and calmodulin-dependent protein kinase II (CAMKII), but not STAT1 signalling (Di Filippo *et al.*, 2016). Another potential neuronal damage mediator is soluble TRAIL released by $CD4^+$ Th cells, which provides a contact-independent pathway of $CD4^+$ T cell-mediated cytotoxicity (Kayagaki *et al.*, 1999) and is known to worsen EAE disease outcome (Aktas *et al.*, 2005).

1.10.3 Neuron-glia interactions in the CNS.

While $CD4^+$ and $CD8^+$ T cells directly interact with neurons via MHC-dependent and -independent mechanisms as well as cytokines, these immune cells can also be involved in organizing, controlling and/or regulating cells that cause neuronal death or protection (Figure 1.11). These cells include microglia, astrocytes as well as oligodendrocytes. Whilst much is known about the antigen presenting function of these particular cells, not much research has been performed on T cell-mediated glial activation or suppression in order to induce or prevent neurodegeneration. In a PD mouse model, T cell accumulation within the substantia nigra pars compacta leads to the suppression of microglial activation and an increase in astrocyte-associated glial cell line-derived neurotrophic factor, resulting in protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurodegeneration (Benner *et al.*, 2004). In agreement, $CD4^+CD25^+$ Tregs adoptive transfer in MPTP-intoxicated mice protects neurons in the nigrostratial system via changes in microglial responses, which upregulate glial cell-derived neurotrophic factors and TGF-β (Reynolds *et al.*, 2007). Furthermore, $CD4^+CD25^+$ Tregs suppress nitrated α-synuclein-induced microglial activation of ROS and NF-κB, supporting the beneficial role of Tregs in PD-associated microglial-mediated neuroinflammation (Reynolds *et al.*, 2009). In addition to the role of T cells in microglia-induced neuroinflammation in PD, $CD4^+CD25^+$ Tregs have been shown to attenuate microglia inflammation, thereby preventing neurodegeneration in a HIV-1 encephalitis mouse model (Liu *et al.*, 2009). How these Tregs induce changes in microglial activation is still unclear. However, it has been shown that $CD4^+CD25^+FoxP3^+$ Treg-derived IL-4 supresses toxic microglial properties in a Cu^{2+}/Zn^{2+} superoxide dismutase-induced amyotrophic lateral sclerosis (ALS) mouse model (Beers *et al.*, 2011). In contrast to the microglial-induced neuroprotection by Tregs, Aβ-specific Th1 cell-derived IFN-γ promotes microglial activation, which significantly accelerates plaque burden in APP/PS1 mice (Browne *et al.*, 2013).

Increases in murine primary cortical astrocyte-induced neuroprotective thiol, including cysteine, glutathione, and cysteinyl-glycine, and lactate is mediated by T cell-derived glutamate and results in a reduction in neuronal apoptosis (Garg, *et al.*, 2008). Furthermore, Th2-derived IL-4 increases BDNF mRNA levels in mouse primary astrocytes (Derecki *et al.*, 2010). The proinflammatory cytokine IFN-γ, however provides neuroprotection during LPS-induced acute neuroinflammation through the induction of astrocyte-secreted IL-6 via STAT3 and ERK signalling pathways (Sun *et al.*, 2017).

In addition to T cell interaction with microglia and astrocytes, OT-I cytotoxic $CD8^+$ T cell caused simultaneous caspase-3 activation in oligodendrocytes in a strictly antigen-dependent manner and collateral neuronal apoptosis was observed due to a spill over of perforin and granzymes (Göbel *et al.*, 2009; Sobottka *et al.*, 2009). Furthermore, antigen-dependent glutamate release from cytotoxic $CD8^+$ T cells causes collateral excitotoxic neuronal cell death during an oligodendrocyte-directed CD8+ T cell attack in the CNS gray matter of acute brain slices (Melzer *et al.*, 2013).

1.11 Lymphocyte-derived IL-16 in the CNS.

In addition to the classical cytokines involved in T cell contact-independent interactions with neurons, our lab has recently shown that lymphocytes are neuroprotective under our experimental conditions by significantly reducing KA-induced toxicity and OGD-induced cell death in OSCs, with a pivotal role for the cytokine IL-16 (Shrestha *et al.*, 2014). In this study, it was investigated whether lymphocytes are protective/detrimental in *in vitro* models of excitotoxicity. Interestingly, exposure of OSCs to lymphocytes reduced neuronal death following both kainate- and OGD-induced neuronal cell death. Similar results were achieved with conditioned medium from lymphocytes but not with boiled conditioned medium, indicating that soluble mediators underlie the observed neuroprotection. Using a cytokine

microarray, IL-16 was the only cytokine upregulated in the lymphocyte conditioned medium, suggesting an interesting role for IL-16 involved in mediating neuroprotection.

IL-16 was initially described as a lymphocyte chemoattractant factor (LCF) secreted by lymphocytes and epithelial cells (Center and Cruikshank, 1982; Cruikshank and Center, 1982) and is now well-known as a proinflammatory cytokine that attracts CD4 receptor containing cells in inflammatory conditions (Center *et al*., 1996; Nicoll *et al.*, 1999). Other immune cells have been reported to release IL-16, including monocytes (Elssner *et al.*, 2004), eosinophils and mast cells (Laberge *et al.*, 1999), DCs (Kaser *et al.*, 1999), B cells (Sharma, *et al*., 2000) and microglia (Guo *et al.*, 2004). It is generated as a precursor protein called pro-IL-16 in the cytosol and is then enzymatically cleaved by caspase 3, releasing bioactive IL-16 (Zhang *et al.*, 1998). IL-16 is the only cytokine that contains PDZ domains, which allow for protein-protein interactions (Wilson *et al.*, 2003) and are located in the protein's C-terminal region. In T cells, IL-16 is known to induce $[Ca^{2+}]_i$ rises, $p56^{lck}$ auto-phosphorylation, IL-2Rα expression and modulates T cell proliferation and cytokine production (Cruikshank *et al.*, 1987, 1991; Ryan *et al.*, 1995). In addition to being a proinflammatory cytokine, it is suggested to have anti-inflammatory properties in rheumatoid arthritis (Klimiuk *et al*., 1999). Indeed, IL-16 can inhibit CD3-dependent lymphocyte activation and proliferation (Cruikshank *et al.*, 1996). However, the exact role of IL-16 and its effect on CNS parenchymal cells is fairly unknown. It has been shown though that IL-16 is upregulated in an EAE mouse model (Skundric *et al.*, 2005). In here, elevated levels of IL-16 along with an increase in active-caspase-3 and $CD4^+$ T cell infiltration was correlated with clinically active disease stages. This suggests that IL-16 does not directly interact with neurons but is involved in $CD4^+$ Th1 cell homing, which then affects neurons (Skundric *et al.*, 2006). By treating EAE mice with anti-IL-16 antibodies, paralysis was successfully reversed and relapses were ameliorated (Skundric *et al.*, 2005). Furthermore, IL-16 has been associated with patients following focal cerebral infarctions, where it was expressed in brains by infiltrating immune cells, including neutrophils, $CD8^+$ T

cells and activated $CD68^+$ microglia/macrophages, which are accumulated in lesion sides (Schwab *et al.*, 2001). In human foetal brains, IL-16 is also expressed by microglia at the 11^{th} gestational week, suggesting a regulatory involvement of IL-16-producing microglia in neuronal development (Schwab *et al.*, 2001). Activated microglia/macrophages also induce IL-16 expression in the early post injury immune response in rats that sustained spinal cord injury, leading to microvessel clustering and secondary damage progression (Mueller *et al.*, 2006). Another emerging role for IL-16 is discovered recently in mild- and moderate-AD patients, but not in severe-AD patients (Motta *et al.*, 2007). This was supported by another study where IL-16 levels were increased in ischemic cerebrovascular dementia and AD patients (Di Rosa *et al.*, 2006). Hence, several studies have shown a correlation of IL-16 with neurological disorders, including EAE (Skundric *et al.*, 2005), MS (Skundric *et al.*, 2015), spinal cord injury (SCI, Bank *et al.*, 2015) and AD (Trombetta *et al.*, 2018) in addition to our work on lymphocyte-derived IL-16-mediated neuroprotection (Shrestha *et al*., 2014).

Next to the immune system-derived IL-16, a neuronal variant of IL-16 (NIL-16) has been discovered in the CNS, which is only expressed in hippocampal and cerebellar post-mitotic neurons (Kurschner and Yuzaki, 1999). The N-terminal portion of this variant includes a novel protein sequence that has two additional PDZ domains. NIL-16 is cleaved via caspase 3 activation in a similar manner to immune cell-derived IL-16. CGN exposure to bioactive IL-16 results in CD4-dependent upregulation of the transcription factor cFos (Fenster *et al.*, 2010) , requiring tyrosine phosphorylation but not p38-MAPK (Kurschner & Yuzaki., 1999). This is in agreement with the IL-16-mediated neuroprotective effect we observed in our laboratory that included inhibition in p38-MAPK phosphorylation (Shrestha *et al*., 2014). Furthermore, IL-16 has been shown to enhance neurite outgrowth, irrespective of CD4 expression (Fenster *et al*., 2010). In addition to serving as a signalling molecule after caspase-3-induced secretion, NIL-16 has been implicated to be involved in the targeting and clustering of neurotransmitter receptors. Using a yeast two-hybrid approach, it was shown that the N-terminal region of NIL-

16 interacts with several ion channel proteins, including NR2A-D, inward rectifier K^+ channels (Kir2.1, 2.3 and Kir4.1, 4.2), the Ca^{2+} channel α1C subunit and A-type K^+ channel subunits (Kv4.1–3, Kurschner & Yuzaki, 1999). Indeed, NIL-16 co-expression with Kv4.2 in COS-7 cells induces Kv4.2 to form dense intracellular clusters and results in a significant reduction in whole-cell A-type current densities (Fenster *et al.*, 2007). However, despite these findings, knowledge of if and how IL-16 modulates CNS function is still very limited and further research is required.

1.12 Microfluidic platforms as a tool to study neuroinflammation.

To be efficiently able to monitor the functional connectivity of neuronal networks and sequentially allow for the isolated study of cells with the confounding influences of a dynamic living environment, our laboratory utilises microfluidic platforms (Robertson, Bushell and Zagnoni, 2014; Samson *et al.*, 2016; MacKerron *et al.*, 2017). In addition, our laboratory has also used these platforms to screen biopsy-derived spheroids from cancer patients (Mulholland *et al.*, 2018) as well as the dynamics of nanoparticle uptake and their consequential effects on DCs (Cunha-Matos *et al.*, 2016). As microfluidics is a technology that uses and manipulates small fluid volumes (10^{-9} to 10^{-18} litres) within microchannels (Whitesides, 2006), it was initially a technology used to miniaturise liquid sample analysis, allowing precise diffusive and convective flows, whilst reducing costs and assay time. Nowadays, it has been widely developed to unravel biological questions, especially within the field of neuroscience (Neto *et al.*, 2016; Osaki *et al.*, 2017; Fantuzzo *et al.*, 2018; Wnorowski, Yang and Wu, 2018). More recently, microfluidic devices are used to address the complex issues of co-culturing multiple cell types to unravel cell-cell interactions (Li *et al.*, 2016). As such, compartmentalised circular microfluidic platform has been used to study neuron/axon-glia interactions (Hosmane *et al.*, 2010; Majumdara *et al.*, 2011). Furthermore, they have been used to examine tau hyperphosphorylation in AD by creating a connected healthy and diseased network (Kunze *et al.*, 2011). Studies have also shown the use of microfluidic platforms comprised of brain

endothelial cells, neurons, astrocytes and microglia in order to recreate the BBB (Brown *et al.*, 2015; Koo, Hawkins and Yun, 2018) or to study a neuromuscular co-culture network (Zahavi *et al.*, 2015). Beyond the world of neuroscience, microfluidic devices have also been used to study the cross-talk between immune cells and cancer (Mattei *et al.*, 2014; Parlato *et al.*, 2017) and the role of TGF-β signalling in tissue injury using a co-culture of two liver cell types (Qing Zhou *et al.*, 2015). Whilst one study investigated the neuroinflammatory mechanisms of AD using a microfluidic platform containing neurons, microglia and astrocytes (Park *et al.*, 2018), to the best of my knowledge no attempts have been made to study the interaction between immune cells and CNS parenchymal cells using these microfluidic devices.

1.13 Working hypothesis and aims.

Whilst the CNS is no longer considered an immune privileged site, many questions remain regarding the role of the immune system in CNS homeostasis and in many neurological diseases, including meningitis, MS, stroke, AD and SCI (Engelhardt, Vajkoczy and Weller, 2017; Klein and Hunter, 2017; Stephenson *et al.*, 2018). Both the innate and adaptive immune system constantly survey the CNS to detect and mount immune responses towards infections and sterile injuries. In addition to T cell-induced immunological surveillance in order to limit immune pathology in the CNS, T cells can be the primary driver of disease or represent a tissue response to neurodegeneration (Wraith and Nicholson, 2012). Hence, many attempts have been made to describe either this pathogenic or neuroprotective role of T cells in the CNS, however there has been discussion about precise function(s) and mechanism(s) of these responses during neuroinflammatory diseases. What is known is that T cells can enter the CNS compartment, including the CSF, perivascular space and CNS parenchyma via different routes, especially under neuroinflammatory conditions. Once in the CNS parenchyma, T cells are reactivated by APCs, including DCs, microglia and astrocytes or following a contact-dependent interaction with neurons in order to exert their effector functions, including cytokine or perforin/granzyme release or recruitment and activation of other inflammatory

cells. Whilst it is known that the inflammatory environment determines T cell effector functions, it is still unclear what drives a T cell to migrate to certain areas in the brain, halt its motility and form an immunological synapse with neurons in the CNS. Furthermore, it is unknown how the contact-dependent and -independent interactions between neurons and T cells may be regulated or even simultaneously cross-regulate final T cell function and neuronal viability. In addition, given the (limited) presence of naïve T cells in the CNS, but the lack in knowledge regarding their function, a better understanding of the interplay between neurons and naïve T cells is required in order to develop drugs that possibly protect against neurodegeneration. Hence, the new and fascinating technology of microfluidics can provide us with a better understanding of the conditions through which neuronal and lymphocyte activity and their interactions are promoted or altered in cell co-culture studies. Therefore, in this study, it is **hypothesized** that neuronal-lymphocyte interactions lead to altered hippocampal network activity. Hence, the **main aim** of this study is to investigate the contact-dependent and -independent effect(s) of lymphocytes on neuronal as well as astrocytic activity and synaptic communication under normal conditions. The following sub aims will be used to test the hypothesis (Figure 1.13):

1. Investigate whether lymphocytes are viable and functional in the same medium as hippocampal neurons and astrocytes required for optimal co-culture experiments.
2. Determine whether the soluble mediator IL-16 changes neuronal and/or astrocytic activity in a contact-independent manner.
3. Establish whether lymphocytes affect neuronal and/or astrocytic activity and synaptic communication in a contact-dependent manner.
4. Determine whether alterations in neuronal activity or neuronal apoptosis/necrosis influence lymphocyte migration.

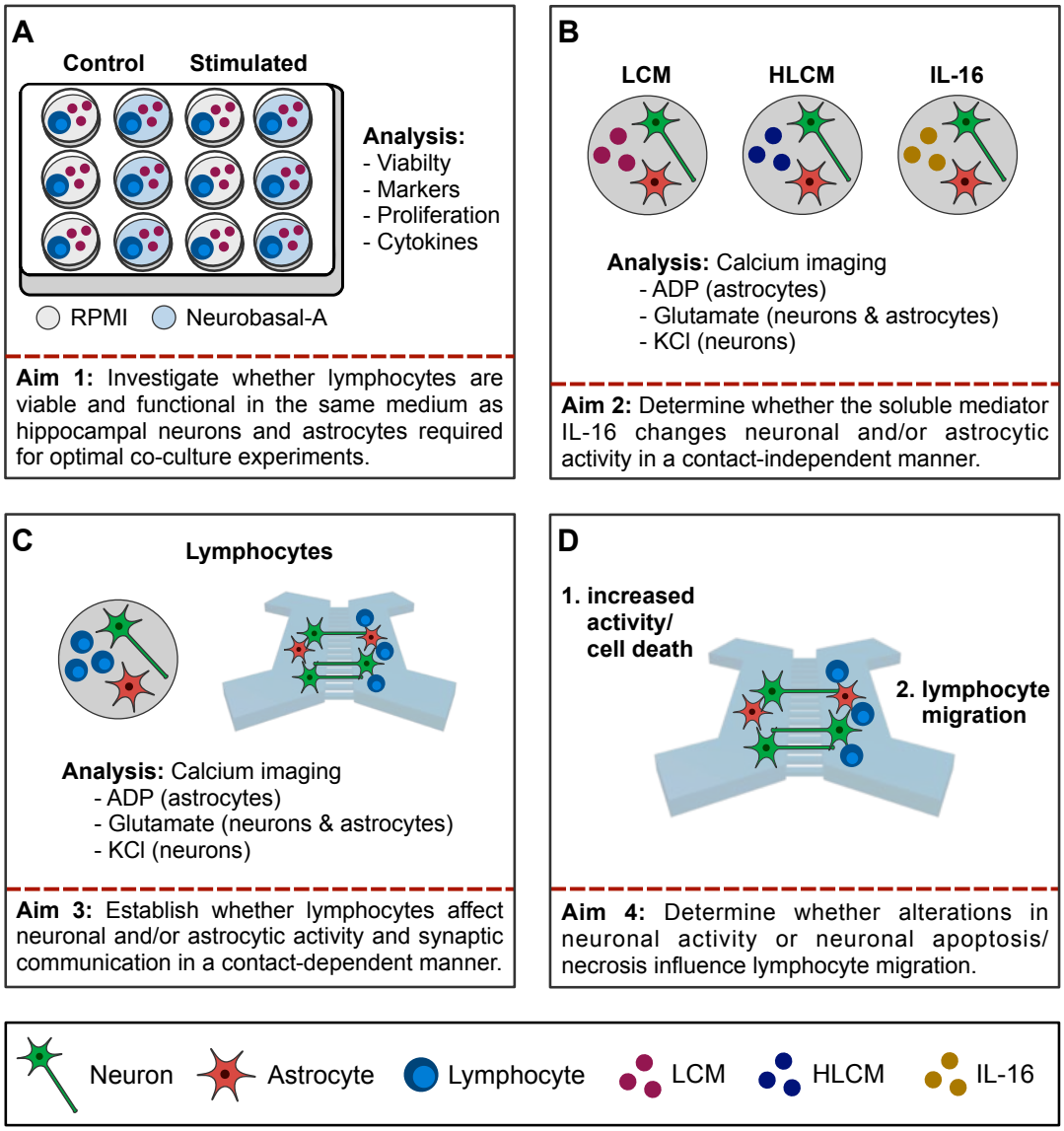

Figure 1.13. Schematic overview of the different research aims. (A-D) Four different research aims will be investigated to test the hypothesis that neuronal-lymphocyte interactions lead to altered hippocampal network activity. Aim 1 will be examined in chapter 3, aim 2 in chapter 4 and aim 3 & 4 in chapter 5.

Chapter 2

Methods.

2.1 Materials.

Chemicals	Supplier	Catalog no.
Adenosine 5′-triphosphate magnesium salt (MgATP)	Sigma-Aldrich, UK	A9187
Adenosine 5′-diphosphate (ADP) sodium salt	Sigma-Aldrich, UK	A2754
Calcium chloride ($CaCl_2$)	Thermo Fisher, UK	10171800
D-(+)-Glucose	Sigma-Aldrich, UK	G7528
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, UK	ED2SS
Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich, UK	E8145
Guanosine 5′-triphosphate sodium salt (NaGTP)	Sigma-Aldrich, UK	G8877
HEPES	Sigma-Aldrich, UK	H3375
L-Glutamic acid	Sigma-Aldrich, UK	G1251
Magnesium chloride ($MgCl_2$) hexahydrate	VWR, UK	25108260
Magnesium sulphate ($MgSO_4$) heptahydrate	Thermo Fisher, UK	10264630
Monosodium dihydrogen phosphate (NaH_2PO_4)	Sigma-Aldrich, UK	71505
Potassium chloride (KCl)	VWR, UK	26764298
Potassium Methanesulfonate ($KMeSO_3$)	Sigma-Aldrich, UK	83000
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich, UK	S5761
Sodium chloride (NaCl)	Sigma-Aldrich, UK	71376
Bovine serum albumin (BSA)	Sigma-Aldrich, UK	A3294
Calcein	Sigma-Aldrich, UK	C0875
CellTracker Red CMTPX Dye	Life Technologies, UK	C34552
Cytosine β-D-arabinofuranoside hydrochloride	Sigma-Aldrich, UK	C6645
Fluo-8	Abcam	Ab142773
Fura-2 AM	Life Technologies, UK	F1221
Papain from papaya latex	Sigma-Aldrich, UK	P4762
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich, UK	P4417
Poly-L-lysine (PLL) hydrobromide	Sigma-Aldrich, UK	P1399
Solutions	**Supplier**	**Catalog no.**
FACSFlow sheath fluid	BD Biosciences, UK	342003
Fixation and permeabilisation solution	BD Biosciences, UK	554722
HBSS	Gibco, UK	14185-045
H_2SO_4 stop solution	Fluka, UK	35276
Methanol >99.8%	VWR, UK	20847.307
Perm/wash buffer	BD Biosciences, UK	554723
PFA	Alfa Aesar	J61899
TMB Microwell Peroxidase Substrate Kit (2 Component)	KPL Insight Biotechnology, UK	5120-0047

Triton X-100 solution	Sigma-Aldrich, UK	93443
Tween-20	Sigma-Aldrich, UK	P1379
Acidic Soluble Collagen 01APA006	Collagen Solutions, UK	FS22007
B-27 Supplement (50x), serum free	Gibco, UK	17504-044
L-glutamine	Sigma-Aldrich, UK	G7513
Neurobasal-A medium	Gibco, UK	10888-022
Neurobasal-A medium w/o phenol red	Gibco, UK	12349-015
Penicillin-streptomycin (P/S)	Gibco, UK	15140-122
Poly-L-Ornithine (PLO) solution	Sigma-Aldrich, UK	P4957
RPMI without L-glutamine	Lonza, UK	BE12-167F
SYLGARD™ 184 Clear Silicone Encapsulant 1.1Kg Kit	Silmid ltd, UK	SG18400110
Antibodies and proteins	**Supplier**	**Catalog no.**
Armenian hamster anti-mouse CD11c, PE	eBiosciences, UK	12-0114-82
Hoechst 33342, Trihydrochloride, Trihydrate	Life Technologies, UK	H3570
Mouse anti-mouse CD45.2, allophycocyanin (APC)	eBiosciences, UK	17-0454-81
Mouse anti-mouse MHC Class I (H-2Kb), Fluorescein (FITC)	eBiosciences, UK	11-5958-80
Propidium Iodide	Sigma-Aldrich, UK	P4170
Rat anti-mouse/human B220 (CD45R), PE	eBiosciences, UK	12-0452-83
Rat anti-mouse CD3, Alexa Fluor (AF)-488	BioLegend, UK	100212
Rat anti-mouse CD4, FITC	eBiosciences, UK	11-0042-85
Rat anti-mouse CD4, Peridinin Chlorophyll Protein Complex (PerCP)	BD Pharmingen, UK	553052
Rat anti-mouse Ki-67, PerCP-Cy5	eBiosciences, UK	46-5698-82
Rat anti-mouse MHC Class II (I-A/I-E), FITC	eBiosciences, UK	11-5321-85
Rat anti-mouse IFN-γ	BD Pharmingen, UK	559065
Recombinant mouse IFN-γ standard	R&D systems, UK	485-MI-100
Rat anti-mouse IFN-γ, Biotin	BD Pharmingen, UK	554410
Rat anti-mouse IFN-γ	eBiosciences, UK	14-7313-85
Recombinant mouse IFN-γ standard	eBiosciences, UK	39-8311-60
Rat anti-mouse IFN-γ, Biotin	eBiosciences, UK	13-7312-85
Rat anti-mouse IL-16	R&D systems, UK	DY1727
Recombinant mouse IL-16 standard	R&D systems, UK	DY1727
Goat anti-mouse IL-16, Biotin	R&D systems, UK	DY1727
Rat anti-mouse IL-2	eBiosciences, UK	14-7022-68
Recombinant mouse IL-2 standard	eBiosciences, UK	39-8021-60
Rat anti-mouse IL-2, Biotin	eBiosciences, UK	33-7021-68
Rat anti-mouse/rat IL-17α	eBiosciences, UK	14-7175-85
Recombinant mouse IL-17α standard	eBiosciences, UK	14-8171-62

Rat anti-mouse/rat IL-17α, Biotin	eBiosciences, UK	13-7177-85
Streptavidin, horse radish peroxidase (HRP)	BD Pharmingen, UK	554066
Chicken anti-mouse/rat MAP2	Merck Millipore, UK	AB5543
Chicken anti-mouse GFAP	Merck Millipore, UK	AB5541
Mouse anti-mouse GFAP	Cell Signaling, UK	3670S
Mouse anti-rat/human Synaptophysin	Merck Millipore, UK	MAB329
Rabbit anti-mouse ß-III-Tubulin	Sigma-Aldrich, UK	T2200
Rabbit anti-mouse CD45	Abcam, UK	Ab10558
Rat anti-mouse CD4	eBiosciences, UK	14-0042-85
Chicken anti-rat AF-488	Life Technologies, UK	A-21470
Donkey anti-mouse AF-555	Life Technologies, UK	A-31570
Goat anti-chicken AF-488	Life Technologies, UK	A-11039
Goat anti-chicken AF-555	Life Technologies, UK	A-21437
Goat anti-rabbit AF-488	Life Technologies, UK	A-11008
Goat anti-rabbit AF-555	Life Technologies, UK	A-21428
Albumin from chicken egg white	Sigma-Aldrich, UK	A5503
Recombinant mouse CCL19/MIP-3ß protein, CF	R&D systems, UK	440-m3-025
Recombinant mouse IL-16 protein	Invitrogen, UK	RP-8610

2.2 Animals.

C57BL/6J, BALB/cJ and homozygous OT-II transgenic mice (backcrossed on a C57BL/6J background) were bred and maintained in the Biological Procedures Unit (BPU) at the University of Strathclyde, Glasgow. OT-II mice, carrying a transgene that encodes for the α- and β-chain TCR on $CD4^+$ Th cells that is specific for chicken $ovalbumin_{323-339}$ (Barnden *et al.*, 1998), were originally purchased from Charles River Laboratories. These OT-II mice are widely used to study T cell biology, including T cell activation. Therefore, these mice were used as a proof-of-principle model to study lymphocyte viability and functionality in serum-free NB-A medium (see chapter 3). All mice were sacrificed in accordance with the UK Home Office Schedule 1 guidelines under the authority of the UK Animals (Scientific Procedures) Act, 1986 and approved by the University of Strathclyde Animal Welfare and Ethical Board.

2.3 Methods.

2.3.1 Mouse lymphocyte preparation.

C57BL/6J, BALB/cJ or OT-II mice (8-10 weeks old, male) were sacrificed via cervical dislocation. Popliteal, inguinal, axillary, brachial, superficial cervical and mesenteric lymph nodes were harvested and placed into complete Roswell Park Memorial Institute (cRPMI) medium containing fetal bovine serum (FBS, 10% v/v), L-glutamine (2 mM) and penicillin/streptomycin (P/S, 100 μg mL^{-1}). Lymphocytes were extracted by tissue homogenisation through a cell strainer (70 μm, Corning Falcon, UK) using a syringe plunger obtaining a single cell suspension. Cell suspensions were then washed and re-suspended in cRPMI medium and plated at a density of $5x10^6$ cells mL^{-1} into 12-well plates (1 mL $well^{-1}$) and maintained in a humidified incubator at 37 °C/5% CO_2 for 18 hours, unless stated otherwise.

2.3.2 Lymphocyte viability and subset assay.

Lymphocyte cell suspensions were prepared in cRPMI or complete Neurobasal-A medium (cNB-A medium) containing B-27 serum-free supplement (2% v/v) and L-glutamine (2 mM) and plated into 12-well plates (see section 2.3.1). Both lymphocyte viability and lymphocyte subsets were analysed using flow cytometry (see section 2.3.7). Lymphocyte viability was checked at different time points during the culture (0-72 hours) using propidium iodide (PI) staining. Lymphocyte subsets were identified using the cell surface markers CD45.2 (leukocytes), B220 (B cells), CD3 (T cells) and CD4 (Th cells) after 18 hours *in vitro*, as this is the time point used for future hippocampal-lymphocyte co-culture experiments. All experiments were performed in triplicate from three independent cultures derived from three different OT-II mice.

2.3.3 Lymphocyte *in vitro* stimulation.

To determine lymphocyte functionality including T cell activation and cytokine secretion, lymphocytes from OT-II mice were first prepared in either cRPMI medium or cNB-A medium and plated in 12-well plates (see section 2.3.1). These cell suspensions were then treated with either vehicle (medium, non-stimulated control condition) or ovalbumin$_{323-339}$ peptide (1 mg mL^{-1}, stimulated condition) and maintained in a humidified incubator at 37 °C/5% CO_2 for 72 hours. Following 72 hours *in* vitro, cells were checked for their CD4 surface marker and intracellular Ki-67 proliferation marker using flow cytometry (see section 2.3.7). Furthermore, IFN-γ, IL-5, and IL-16 secretion by the cultured cells was analysed using enzyme-linked immunosorbent assays (ELISAs) (see section 2.3.8). All experiments were performed in triplicate from four independent cultures derived from four different OT-II mice.

2.3.4 $CD4^+$ T helper cell isolation.

To determine IL-16 release from non-stimulated and stimulated $CD4^+$ Th cells, ovalbumin-specific $CD4^+$ Th cells were isolated using the negative selection mouse $CD4^+$ T cell isolation kit according to manufacturer's instructions (Miltenyi Biotec, UK). In brief, the spleen was harvested in addition to lymph nodes to increase lymphocyte cell count. Thus, single cell suspensions were prepared from lymph nodes and spleen from OT-II mice in cNB-A medium (see section 2.3.1). Following cell counts, 10^8 total cells were re-suspended in Hank's balanced salt solution (HBSS, 400 µL), and a Biotin-Antibody Cocktail (100 µL) consisting of biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHC-class II, Ter-119 and TCRγ/δ was added to the cells. Following a 5-minute incubation at 4 °C, HBSS (300 µL) and Anti-Biotin Microbeads (200 µL) were added to magnetically label the cells. Cells were then mixed by carefully pipetting up and down, followed by a 10-minute incubation at 4 °C before the total cell suspension was applied onto a MACS® column (LS Column, Miltenyi Biotec, UK) placed in the magnetic field of a MACS® separator (Miltenyi Biotec, UK). The magnetic field retained the magnetically labelled non-target cells in the column, while the target non-labelled $CD4^+$ Th cells flowed through the column into a collection tube (15 mL). The column was then washed with HBSS (3 mL) to collect the remaining $CD4^+$ Th cells. Subsequently, the column was removed from the MACS® separator and placed into a new collection tube (15 mL) to flush out the magnetically labelled non-$CD4^+$ cells by firmly pushing the plunger into the column. Enriched $CD4^+$ Th cell suspensions, $CD4^+$ depleted cell suspensions and non-isolated control cell suspensions were re-suspended in cNB-A medium at a density of $5x10^6$ cells mL^{-1} for further use in *in vitro* stimulation experiments.

2.3.5 Mouse bone-marrow derived dendritic cell preparation.

Mouse bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6J mice (8-10 weeks old, male) as described previously (Lutz *et al.*, 1999; Cunha-Matos *et al.*, 2016). Hence, C57BL/6J mice were sacrificed via cervical dislocation and their tibias and femurs were harvested with the epiphyses removed. A needle (26 Gauge) was used to flush out and dissociate the bone marrow to form a single cell suspension in cRPMI medium (10 mL) supplemented with medium derived from x63 cells expressing granulocyte-macrophage colony-stimulating factor (GM-CSF, 10% v/v). This single cell suspension was seeded into a bacteriological petri dish and maintained in a humidified incubator at 37 °C/5% CO_2 for 7 days. At 3 days *in vitro* (DIV), fresh cRPMI medium (10 mL) supplemented with GM-CSF (10% v/v) was added, whilst at 5 DIV, half of the medium was replenished with fresh cRPMI medium supplemented with GM-CSF (10% v/v). Matured BMDCs were harvested at 7 DIV by collecting the medium containing floating BMDCs as well as adhering BMDCs using a syringe plunger (1 mL) as a scraper. Cells were washed, counted and re-suspended at a density of $1x10^6$ cells mL^{-1} in cNB-A medium for further use as APCs in *in vitro* stimulation experiments of isolated $CD4^+$ Th cells. At time of harvesting, BMDCs were checked for the DC specific cell surface marker CD11c and MHC I or MHC II using flow cytometry (see section 2.3.6). BMDC preparations were shown to be on average 70% $CD11c^+$, 70% MHC I^+ and 50% MHC II^+ (Figure 2.1).

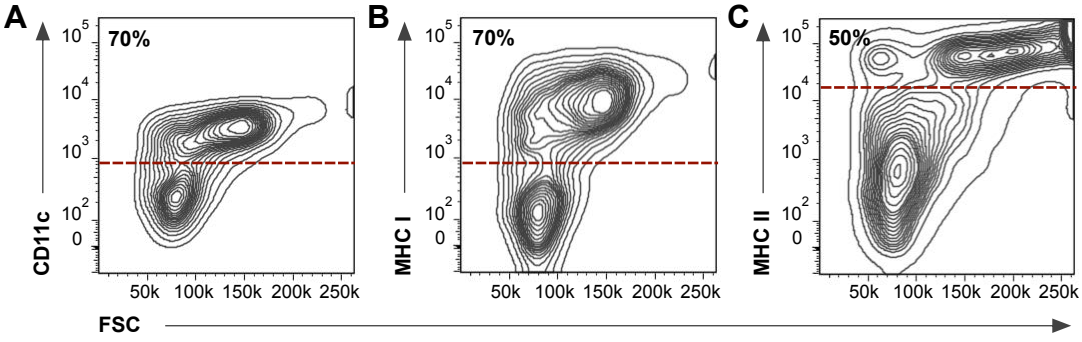

Figure 2.1. Representative flow cytometry plots revealing BMDC (A) CD11c, (B) MHC I and (C) MHC II surface expression at 7 DIV. Data is a representative of three BMDC cultures.

2.3.6 $CD4^+$ T helper cell *in vitro* stimulation.

Non-isolated control cell suspensions, enriched $CD4^+$ Th cell suspensions and $CD4^+$ depleted cell suspensions from OT-II mice were prepared in cNB-A medium (see section 2.3.4). Cells were plated into 24-well plates (0.5 mL $well^{-1}$) at a density of $2.5x10^6$ cells mL^{-1} and treated with either vehicle (medium, non-stimulated control condition), BMDCs ($5x10^5$ cells mL^{-1}, non-stimulated control condition) or $ovalbumin_{323-339}$ peptide plus BMDCs (1 mg mL^{-1}, $5x10^5$ cells mL^{-1}, stimulated condition) and maintained in a humidified incubator at 37 °C/5% CO_2 for 72 hours. Cells were then checked for their CD4 surface marker using flow cytometry (see section 2.3.7). Furthermore, IFN-γ, IL-2, IL-17α and IL-16 secretion by the cultured cells was analysed using ELISAs (see section 2.3.8). All experiments were performed in triplicate from four independent cultures derived from four different OT-II mice.

2.3.7 Flow cytometric analysis.

Cells were harvested and transferred to a single fluorescence-activated cell sorting (FACS) tube and re-suspended in FACSFlow sheath fluid buffer (100 µL) containing the appropriate fluorescently-conjugated antibodies for the cell surface markers listed in Table 2.1 and incubated for 20 minutes at 4 °C in the dark. In the case of intracellular Ki-67 lymphocyte labelling, cells were fixed and permeabilized using BD Cytofix/Cytoperm Solution (BD Biosciences, UK) for 20 minutes at 4 °C in the dark before the fluorescently-conjugated Ki-67 antibody diluted in BD Perm/Wash buffer (BD Biosciences, UK) was added for 30 minutes at 4 °C in the dark. Subsequently, cells were washed and re-suspended in FACSFlow sheath fluid buffer (300 µL) for data acquisition. Data was acquired using a FACSCanto flow cytometer (BD Biosciences, UK) using FACSDiva Software (BD Biosciences, UK), according to manufacturer's instructions. Before acquiring data, forward scatter (FSC) and side scatter (SSC) voltages were adjusted to gate the cells of interest and antibody-capture beads (BD Calibrite Beads, UK) were used as single-color compensation controls for each reagent used

to correct for spectral overlap. Finally, data was analysed using FlowJo 7.6.1 software (FlowJo, LLC, Oregon, USA).

Table 2.1. Flow cytometry antibodies used for cell marker identification.

Cell marker	Antibody	Dilution
dsDNA	Propidium Iodide	1:300
CD45.2	APC anti-mouse CD45.2	1:300
B220	PE anti-mouse B220	1:300
CD3	AF-488 anti-mouse CD3	1:300
CD4	PerCP anti-mouse CD4 FITC anti-mouse CD4	1:300 1:300
CD11c	PE anti-mouse CD11c	1:300
H-2Kb	FITC anti-mouse MHC Class I	1:300
I-A & I-E	FITC anti-mouse MHC Class II	1:300
Ki-67	PerCP-Cy5 anti-mouse Ki-67	1:200

2.3.8 Enzyme-linked immunosorbent assay.

ELISAs were conducted to quantify cytokine production using mouse IFN-γ antibodies (BD Pharmingen), mouse IFN-γ, IL-2 and IL-17α ready-set-go antibodies (eBiosciences) and mouse DuoSet IL-16 kit according to manufacturer's instructions (R&D systems, UK). Reagents and antibody concentrations used are listed in Table 2.2. Incubation steps were performed at room temperature, except for the IFN-γ antibodies (BD Pharmingen), which were performed in a humidified 37 °C/5% CO_2 incubator according to manufacturer's instructions (R&D systems, UK). In brief, high binding 96-well ELISA plates (Greiner Bio-One, UK) were coated overnight (4 °C) with a primary capture antibody diluted in coating buffer (phosphate buffered saline (PBS), pH 7.4), followed by a wash step using Tween-20 in PBS (0.05%, pH 7.4). Plates were then blocked using blocking buffer for a minimum of 1 hour and washed

afterwards. Standards prepared in the corresponding blocking buffer and samples (freshly collected supernatants, diluted in corresponding blocking buffer when required) were incubated for 2 hours, followed by another wash step. Subsequently, a biotin-conjugated detection antibody was added for 1 hour (2 hours for IL-16 assay according to manufacturer's instructions (R&D systems, UK)), washed and a 30-minute incubation step with HRP-conjugated streptavidin was performed. Following a final wash step, TMB peroxidase substrate solution was added, developing a blue colour reaction. This reaction was stopped with H_2SO_4 and absorbance was measured at 450 nm using the Epoch Microplate Spectrophotometer (BioTek, UK) according to manufacturer's instructions with Gen5 1.10 software (BioTek, UK).

Table 2.2. Reagents and antibody concentrations used in ELISA.

Cytokine	Capture	Blocking	Standard	Detection
IFN-γ [(1)]	Rat anti-mouse IFN-γ (2 μg mL^{-1})	10% FBS in PBS (pH 7.4)	Recombinant mouse IFN-γ (20 ng mL^{-1}) [(3)]	Biotinylated rat anti-mouse IFN-γ (0.5 μg mL^{-1})
IFN-γ [(2)]	Anti-mouse IFN-γ (1 μg mL^{-1})	10% FBS in PBS (pH 7.4)	Recombinant mouse IFN-γ (1 ng mL^{-1})	Biotinylated anti-mouse IFN-γ (1 μg mL^{-1})
IL-2 [(2)]	Anti-mouse IL-2 (2 μg mL^{-1})	10% FBS in PBS (pH 7.4)	Recombinant mouse IL-2 (1 ng mL^{-1})	Biotinylated anti-mouse IL-2 (2 μg mL^{-1})
IL-17α [(2)]	Anti-mouse IL-17α (2 μg mL^{-1})	10% FBS in PBS (pH 7.4)	Recombinant mouse IL-17α (1 ng mL^{-1})	Biotinylated anti-mouse/rat IL-17α (1 μg mL^{-1})
IL-16 [(3)]	Rat anti-mouse IL-16 (4 μg mL^{-1})	1% BSA in PBS (pH 7.4)	Recombinant mouse IL-16 (3 ng mL^{-1})	Biotinylated goat anti-mouse IL-16 (20 μg mL^{-1})

[(1)] BD Pharmingen [(2)] eBiosciences [(3)] R&D systems

2.3.9 Mouse primary hippocampal culture preparation on coverslips.

Primary hippocampal cultures were prepared as previously described (Ritchie *et al.*, 2018). All media and solutions mentioned were filter-sterilized and maintained in a 37 °C water bath until required. Briefly, C57BL/6J mice (1-2 days old) were sacrificed via cervical dislocation and decapitated afterwards. Under sterile conditions, the brain was removed, followed by a separation of both hemispheres. After orientating the medial side of the brain upwards, the cerebellum and brainstem (medulla oblongata, pons and midbrain) were folded backwards exposing the hippocampus, which was then removed using tweezers and placed into HBSS composed of (in mM): NaCl 116, KCl 5.4, $NaHCO_3$ 26, NaH_2PO_4 1.3, $MgSO_4$ 2, EDTA 0.5, D-glucose 25, $CaCl_2$ 1.8. Subsequently, the hippocampi were transferred to a papain solution (papain in HBSS, 1.5 mg mL^{-1}) for 20 minutes at 37 °C, followed by a second dissociation step via trituration in bovine serum albumin (BSA) solution (BSA in HBSS, 10 mg mL^{-1}) using three sterile flame-polished glass Pasteur pipettes of decreasing diameter. Dissociated cells were re-suspended into phenol red-free cNB-A medium. This cell suspension ($5x10^5$ cells mL^{-1}, 100 μL) was seeded onto poly-L-lysine (PLL, 0.01 mg mL^{-1}) coated sterile coverslips (13 mm diameter, VWR, UK) placed into petri dishes (35 mm diameter, Falcon, Scientific Laboratories Supplies, UK) with 3 coverslips per dish. Cells were then placed into a humidified incubator at 37 °C/5% CO_2 for 1 hour to ensure cell adhesion. Once adhered, medium was removed from the coverslips and dishes were filled with phenol red-free cNB-A medium (2 mL), ensuring the coverslips were covered. Cells were maintained in a humidified incubator at 37 °C/5% CO_2 for 11 to 14 DIV, with half the media replaced with fresh phenol red-free cNB-A medium at 5 and 10 DIV. Additionally, cytosine β-D-arabinofuranoside (Ara-C, 1 μM) was added to the cultures at 5 DIV to inhibit glial cell proliferation.

2.3.10 Immunocytochemistry.

Immunocytochemistry was performed on primary hippocampal cultures and lymphocyte preparations as previously described (Abdul Rahman *et al.*, 2016). Hence, cultures were

washed 3 times in PBS and the cells were then fixed in ice-cold paraformaldehyde (PFA, 4% v/v) for 10 minutes, followed by a PBS wash step. Subsequently, cultures were treated with ice-cold methanol (100%) for a further 10 minutes. After washing the cultures, cells were permeabilised for 10 minutes with Triton-X (0.01% v/v) in blocking solution (FBS (5% v/v) and BSA (0.01 g mL^{-1}) in PBS). Non-specific binding sites were then blocked for a further 15 minutes using blocking solution. Primary antibodies diluted in blocking solution (see Table 2.3) were added and incubated overnight in a wet box at 4 °C. Following this, cultures were washed to remove any unbound primary antibody and an appropriate fluorescently-conjugated secondary antibody (Alexa Fluor (AF)-488 or AF-555, 1:200, Life Technologies, UK) prepared in blocking solution was added for 1 hour at room temperature in the dark. Following a final wash step to remove any unbound secondary antibody, cells on coverslips were imaged using an upright epifluorescence microscope (Olympus BX51W, Japan) with either a 20×/0.5w, 40×/0.75w or 60×/0.9w water-immersion objective lens (UMPlanFL, Olympus, Tokyo, Japan) with excitation using appropriate filters to visualise the secondary antibody signals. Fluorescent signals were visualized using a cooled CCD digital camera (01-RET-EXI-F-M-12-C, Q-Imaging, UK) and WinFluor v2.7.8 imaging software (Dempster *et al.*, 2002). Cells in microfluidic devices (prepared as described below, Methods 2.3.15) were imaged using an inverted epifluorescence microscope (Observer Z1, Zeiss, UK) with either a 10×/0.3 Ph1, 20×/0.5 Ph2, 40×/0.75 Ph2 objective lens, or a 63×/1.25 Ph3 oil-immersion objective lens (EC Plan NeoFluar, Zeiss, UK) with excitation using appropriate filters to visualise the secondary antibody signals. Fluorescent signals were visualized using a CMOS digital camera (ORCA-Flash 4.0, model: C11440-22CU, Hamamatsu Photonics, UK) and Zen2 imaging software (Zeiss, UK). Images were then analysed using ImageJ 1.49v software (National Institutes of Health, USA) or Zen 2.5 (blue edition) imaging software (Zeiss, UK) where appropriate.

Table 2.3. Immunocytochemistry antibodies used for cell marker identification.

Cell marker	Primary Antibody	Dilution
Microtubule-associated protein 2	Chicken anti-mouse MAP2	1:500
ß-III-Tubulin	Rabbit anti-mouse ß-III-tubulin	1:500
Synaptophysin	Mouse anti-mouse Synaptophysin	1:500
Glial fibrillary acidic protein	Mouse anti-mouse GFAP Chicken anti-mouse GFAP	1:500
CD4	Rat anti-mouse CD4	1:400
CD45	Rabbit anti-mouse CD45	1:5000

2.3.11 Calcium imaging in primary hippocampal cultures on coverslips.

Live cell Ca^{2+} imaging experiments in hippocampal cultures on coverslips (11-14 DIV) were performed as previously described (Hridi et al., 2019). Cells were washed three times with HBS external solution and then loaded with the ratiometric Ca^{2+}-sensitive dye Fura-2 AM (1 μM) in HBS for 1 hour in the dark at room temperature. Following another wash step, cultures were placed into a perfusion chamber attached to the microscope stage and continuously perfused with HBS (3.5 mL min^{-1}). Baseline Ca^{2+} recordings were obtained prior to drug applications. All drugs were applied via the perfusate including adenosine 5′-diphosphate (ADP, 20 μM, 2 minutes), L-glutamic acid (glutamate, 10 μM, 1 minute), KCl (25 mM, 2 minutes) and recombinant IL-16 (rIL-16, 300 pg mL^{-1}, 5 minutes). To rule out that the drugs were having an effect on each other, the order of drug application was switched around. Cells were visualised using an upright epifluorescence microscope (BX51W, Olympus, Japan) and a 20×/0.5w water-immersion objective lens (UMPlanFL, Olympus, Tokyo, Japan). Ca^{2+} levels and responses from the soma of hippocampal neurons and astrocytes were recorded using a cooled CCD digital camera (01-RET-EXI-F-M-12-C, Q-Imaging, UK) with an exposure time of 500 ms and frame rate of 0.5 Hz. Time-lapse ratiometric images (350/380 nm; OptoLED, Cairn Research, UK) were obtained using WinFluor v2.7.8 imaging software (Dempster *et al.*,

2002). Cells were identified as neurons and astrocytes based on their morphological characteristics and their cell specific response to ADP or high extracellular KCl. Data was analysed offline using WinFluor v2.7.8 imaging software. As such, from each recording, regions of interest (ROI) identifying cell somas were selected and the corresponding mean fluorescence intensity within a ROI was measured for each time point, with the change in fluorescence ratio expressed as $\Delta F/F0$. Multiple responses from the same ROI were included. For Ca^{2+} imaging experiments, *n* is the total number of cells analysed from at least three independent hippocampal cultures derived from three different C57BL/6J mice.

2.3.12 Contact-dependent and -independent lymphocyte treatment of hippocampal cultures on coverslips.

To determine hippocampal-lymphocyte interactions *in vitro*, the contact-dependent and contact-independent effects of lymphocytes on hippocampal excitability were studied using live cell Ca^{2+} imaging (see section 2.3.11). In brief, prior to live cell Ca^{2+} imaging, hippocampal cells were treated (1 hour) with either hippocampal conditioned medium (HCM) control, lymphocytes ($4x10^6$ cells mL^{-1}), two types of lymphocyte conditioned medium (LCM) or rIL-16 (300 pg mL^{-1}) in 4-well plates (1.9 cm^2 $well^{-1}$, Nunc, Thermo Scientific, UK) in a humidified incubator at 37 °C/5% CO_2. Preparation of these treatments is described below (Figure 2.2). Hippocampal cells were simultaneously loaded with Fura-2 AM (1 μM) during treatment, instead of loading in external solution afterwards, thereby avoiding a washout of the lymphocyte effects. Following treatment, cultures were washed three times with HBS and placed into a perfusion chamber attached to the microscope stage, ready for imaging.

2.3.12.1 Hippocampal conditioned medium – vehicle control:

Mouse primary hippocampal cultures were cultured on coverslips from C57BL/6J mice (see section 2.3.9). The culture medium (HCM, 250 μL hippocampal $coverslip^{-1}$ $well^{-1}$) was then used as 'HCM control', a control for lymphocyte treatment.

2.3.12.2 Lymphocytes treatment:

Lymphocyte cell suspensions from C57BL/6J mice were prepared in HCM (see section 2.3.1). Cells were plated at a density of 10^6 cells mL^{-1} into 12-well plates (1 mL $well^{-1}$) and maintained in a humidified incubator at 37 °C/5% CO_2 for 18-22 hours. Cells from each well were then washed and re-suspended in HCM (250 µL) to a density of $4x10^6$ cells mL^{-1} and applied directly to 1 hippocampal coverslip $well^{-1}$. This was referred to as 'lymphocyte treatment'.

2.3.12.3 Hippocampal conditioned medium 18-22 hours – vehicle control:

Mouse primary hippocampal cultures were cultured on coverslips from C57BL/6J mice (see section 2.3.9). Coverslips were removed from the petri dishes the day before experimentation and the dishes with the remaining medium were maintained in a humidified incubator at 37 °C/5% CO_2 for a further 18-22 hours. This medium (250 µL hippocampal $coverslip^{-1}$ $well^{-1}$) was then used as 'HCM control', a control for lymphocyte conditioned medium (LCM) treatment and hippocampal lymphocyte conditioned medium (HLCM) treatment.

2.3.12.4 Lymphocyte conditioned medium treatment:

Lymphocyte cell suspensions from C57BL/6J mice were prepared in HCM (see section 2.3.1) and plated at a density of $4x10^6$ cells mL^{-1} into 12-well plates (1 mL $well^{-1}$) and maintained in a humidified incubator at 37 °C/5% CO_2 for 18-22 hours. The culture medium (250 µL hippocampal $coverslip^{-1}$ $well^{-1}$) was then used as 'LCM treatment'.

2.3.12.5 Hippocampal lymphocyte conditioned medium treatment:

Lymphocyte cell suspensions from C57BL/6J mice were prepared in HCM (see section 2.3.1). Cells were then plated at a density of $4x10^6$ cells mL^{-1} into 12-well plates (1 mL $well^{-1}$) and maintained in a humidified incubator at 37 °C/5% CO_2 for 18-22 hours. Following this, cell suspensions $well^{-1}$ were washed and re-suspended in HCM (1 mL) at a density of $4x10^6$ cells mL^{-1} and added to a hippocampal coverslip $well^{-1}$ for 23-25 hours. Following the 23-25-hour

incubation, the co-culture medium (250 µL hippocampal coverslip^{-1} well^{-1}) was then used as 'HLCM treatment'.

2.3.12.6 Recombinant IL-16:

A stock of rIL-16 was prepared in sterile deionised (DI) water (0.1 µg mL^{-1}) on the day of use and further dissolved into HCM (250 µL hippocampal coverslip^{-1} well^{-1}) to a final concentration of 300 pg mL^{-1}. The activity of rIL-16 (cat# RP-8610) is detectable starting at 5 ng mL^{-1}, however this was determined by chemoattraction of primary human T cells (dose-dependent). 'Denatured rIL-16' was prepared by boiling the daily stock in a 90 °C water bath (1 hour), which was then further dissolved into HCM (3 µL mL^{-1}) similar to rIL-16. Distilled water (3 µL mL^{-1}) in HCM was used as 'vehicle'.

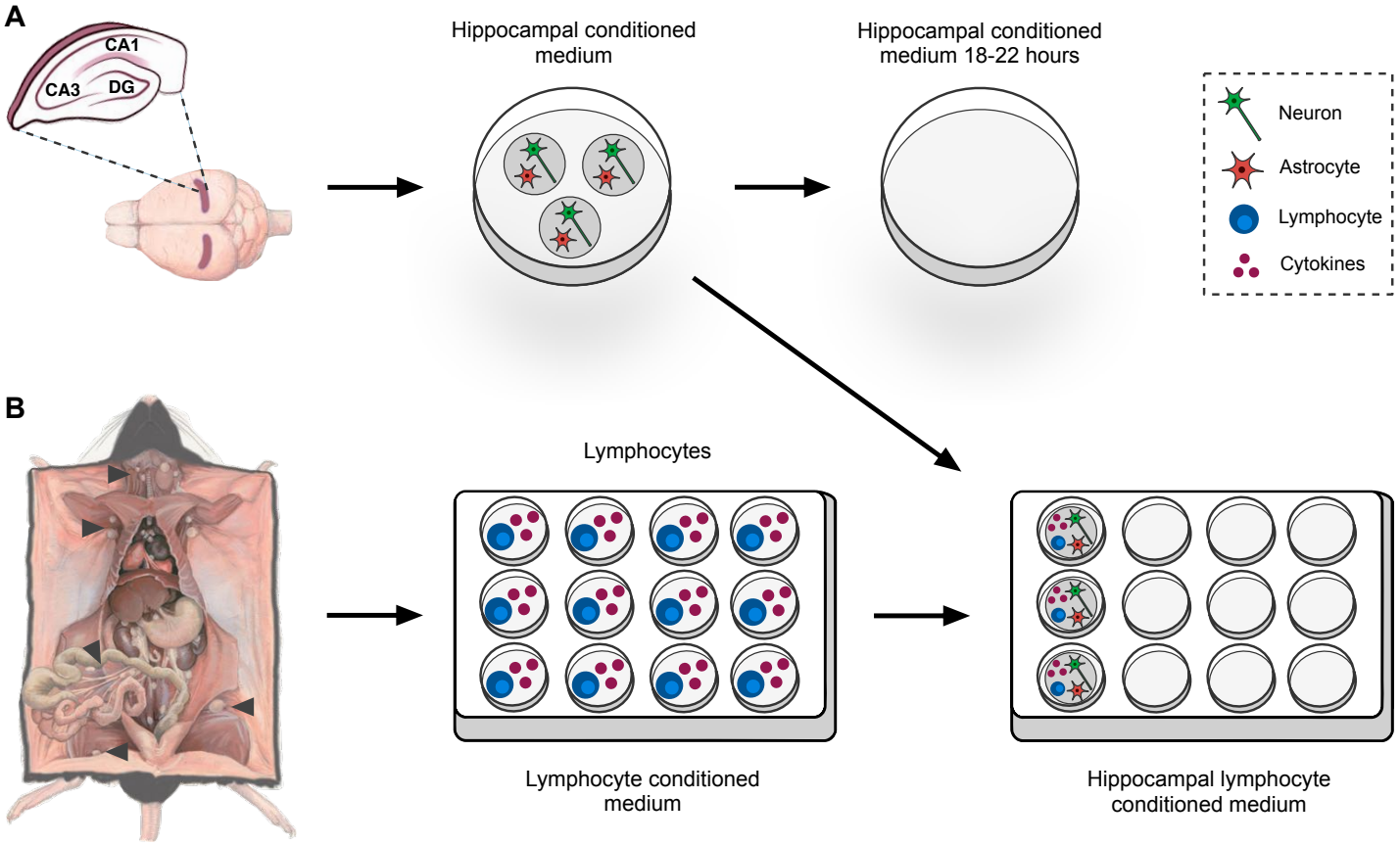

Figure 2.2. Schematic diagram illustrating hippocampal culture treatment preparation. (A) Both vehicle controls, HCM and HCM 18-22 hours, were prepared from C57BL/6J hippocampal culture medium, with or without hippocampal coverslips, respectively. (B) Lymphocytes and LCM were prepared from C57BL/6J lymph nodes. Lymphocytes were added to hippocampal coverslips to create HLCM.

2.3.13 Microfluidic polydimethylsiloxane device preparation.

A silicon master mould was created using standard photolithography techniques and kindly provided by the group of Dr. Michele Zagnoni from the Department of Electronic and Electrical Engineering, University of Strathclyde, Glasgow (Robertson *et al*., 2014). This master mould contains features for the preparation of 6 microfluidic devices (Figure 2.3) and could be used several times using soft-lithography techniques. Polydimethylsiloxane (PDMS, ~5 mm depth) was poured onto the silicon master template to create a replica mould of the patterned microfluidic features. For this, liquid PDMS (Silicone Elastomer Base) was mixed with the corresponding curing agent (10:1 ratio w/w). This was degassed in a desiccator to remove air bubbles, followed by a curing step in a 70-80 °C oven for a minimum of 2.5 hours. The cured PDMS was removed from the master mould using a scalpel and individual devices were then cut out using a scalpel blade. For each device, four through holes were created with a biopsy punch needle (8 mm diameter) to form open wells to fluidically access the two microfluidic chambers (Figure 2.3A).

2.3.14 Bonding of PDMS to glass coverslips.

In order to create fluidically sealed environments, PDMS devices were bonded to a glass coverslip support (40 x 25 mm, VWR, UK). Prior to bonding, both PDMS devices and the glass coverslips were cleaned and made dust-free using scotch tape (Scotch, UK). Bonding was carried out using an oxygen plasma asher (Pico A, Diener Electronic, Germany), which promotes formation of silanol (-Si-OH) groups on the surfaces of the PDMS devices and the glass coverslips. This will enable the formation of covalent bonds (-Si-O-Si-) between contacting PDMS and glass surfaces post oxygen plasma exposure. To achieve this, cleaned PDMS devices and glass coverslips were put into a glass petri dish with their cleaned surface facing upwards, then placed into the oxygen plasma asher and exposed to oxygen plasma for 12 seconds at 50W. Once exposed, devices were taken out of the oxygen plasma asher and

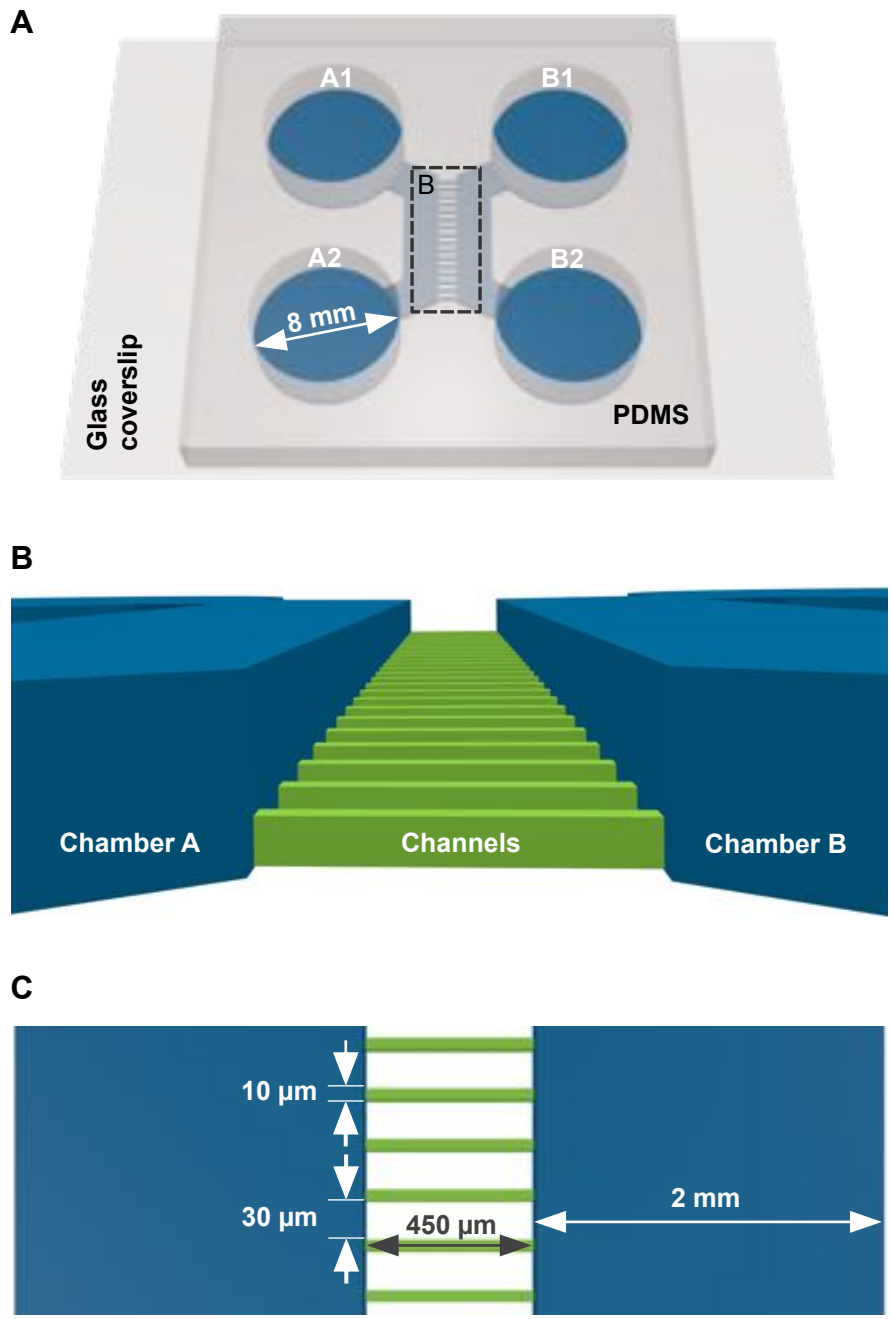

Figure 2.3. Schematic representation of a microfluidic PDMS device. (A) Top view of a fully fabricated PDMS device bonded to a glass coverslip with the fluidic network visible in blue. (B) The first thin layer of the fluidic network consists of an array of microchannels (green) connecting two chambers (A and B, blue) in which cells are seeded via the open wells. (C) Representative image showing dimensions of the microchannels and chamber.

manually placed on the glass coverslips with their features side down, creating a permanent bond. Bonded devices were then put into a glass petri dish and placed back in the oxygen plasma asher. Following a second exposure to oxygen plasma for 2 minutes at 100W, a glass lid was used to cover the glass petri dish, keeping the devices as sterile as possible in order to prevent hippocampal culture contamination in the devices. The exposure to oxygen plasma rendered the interior surfaces temporarily hydrophilic, facilitating device filling with liquids.

2.3.15 Characterisation of drug delivery in microfluidic devices.

To validate that drug delivery to cells was performed without cross-contamination between the two chambers in microfluidic devices, experiments were conducted using calcein, a fluorescent marker (Figure 2.4; Robertson *et al*., 2014). Following bonding of devices to glass coverslips in the oxygen plasma asher, a second exposure to oxygen plasma was not required as devices did not need to be sterile for this experiment. Instead, devices were immediately filled with DI water and kept in the fridge until further use. Prior to experimentation, devices were washed and re-filled with DI water (200 µL well^{-1}) and transferred to the microscope stage. Subsequently, 100 µL DI water was extracted from each of the connecting wells B1 and B2, creating a hydrostatic pressure gradient across the microchannels that induced a small fluid flow from chamber A towards chamber B. At this point baseline recordings (4 minutes) were started prior to calcein application (50 µM final concentration). In order to apply calcein (50 µL) into chamber B via well B1, 50 µL DI water was first extracted from each of the connecting wells B1 and B2. Calcein was then allowed to flow through chamber B for 10 seconds before another application of calcein (50 µL) was administered to well B2. Following a further 4-minute-recording, 150 µL was extracted from each of the connecting wells A1 and A2, reversing the hydrostatic pressure gradient. During the recording, calcein fluorescence was visualized using an inverted microscope (Axio Observer A1, Zeiss, UK) with a 5×/0.12 objective lens (A-Plan, Zeiss, UK) and EMCCD camera (LucaR, Andor Technologies, UK) at a frame rate of 2.0 Hz and 400 ms exposure time. Time-lapse images of the fluorescent signal

temporal evolution were obtained using Andor SOLIS 4.19.30001.0 software (Andor Technologies, UK). From each recording, ROIs were selected in both chambers A and B, proximal and distal to the microchannels. The corresponding mean fluorescence intensity within a ROI in response to calcein was then computed for each time point. Experiments were performed in three independent microfluidic devices.

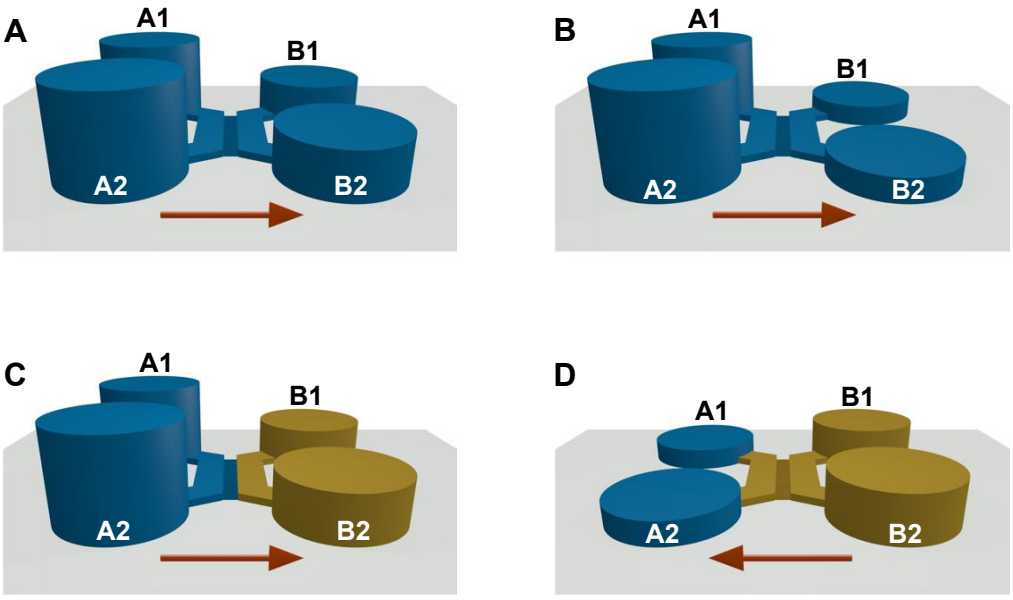

Figure 2.4. Schematic representation highlighting the protocol used for the evaluation of absence of cross-contamination between chambers in microfluidic devices using calcein. (A) A hydrostatic pressure gradient from chamber A towards chamber B (orange arrow) was introduced and maintained during baseline recordings (4 minutes). (B) 50 µL DI water was extracted from well B1 and B2 prior to calcein application. (C) Calcein solution (50 µL) was added to well B1 followed by well B2. (D) Following 4 minutes, 150 µL was extracted from well A1 and A2, reversing the hydrostatic pressure gradient (reversed orange arrow) and forcing calcein to flow into chamber A.

2.3.16 Mouse primary hippocampal culture preparation in microfluidic devices.

Following device bonding to glass coverslips in the oxygen plasma asher, glass petri dishes with microfluidic devices were transferred into a sterile hood and placed into sterile 4-well plates (21.8 cm^2 $well^{-1}$, Nunc, Thermo Scientific, UK) for hippocampal culture preparation. Three out of four wells contained two devices per well and one well was filled with sterile PBS (3 mL) to ensure maximal humidity, preventing significant medium evaporation from devices. Devices were coated immediately with poly-l-ornithine (PLO, 0.01% v/v) for a minimum of 3 hours, which resulted in a better cell adhesion than PLL used for coverslips. Once coated, PLO was washed out using phenol red-free cNB-A medium and devices filled with phenol red-free cNB-A medium were placed into a humidified incubator at 37 °C/5% CO_2 until hippocampal cells prepared from BALB/cJ mice (see section 2.3.9) were ready to be seeded. Following cell preparation, cell suspensions ($5x10^6$ cells mL^{-1}, 10 µL) were loaded into chamber A and B by holding the pipette tip in well A1 and B1 close to the inlet of the chamber. Devices were put back in a humidified incubator at 37 °C/5% CO_2 for 10-15 minutes to ensure cell adhesion. All four wells (200 µL maximum) were then topped up in 50 µL increments, starting with wells A1 and B1, followed by A2 and B2. Cultures were maintained in a humidified incubator at 37 °C/5% CO_2 until ready for experimentation (12-14 DIV), with half the media replaced with fresh phenol red-free cNB-A medium every 2-3 days (Figure 2.5). Medium replacement was performed by extracting 80 µL from all 4 wells, starting with the connecting wells A1 and A2, followed by the connecting wells B1 and B2. Fresh medium (100 µL) was then added to wells A1 and B1, allowing a small medium fluid flow through the corresponding chambers, replenishing the medium in the chambers. Wells A2 and B2 were then topped up with fresh medium (100 µL). Additionally, Ara-C (1 µM) was added to the cultures at 5 DIV to inhibit glial cell proliferation.

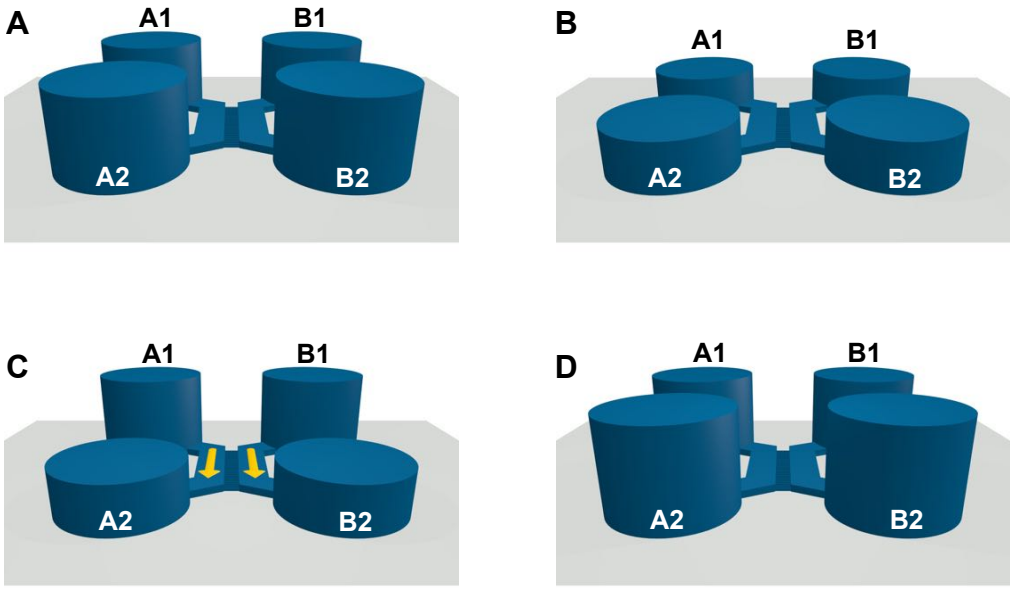

Figure 2.5. Schematic representation highlighting the protocol used for hippocampal culture feeding in microfluidic devices. (A) Schematic representation of the medium volume (~180 µL) in all four wells of a device just before feeding. (B) 80 µL medium was extracted from all four wells prior to the addition of fresh medium. (C) Fresh medium (100 µL) was added to wells A1 and B1 allowing medium to slowly flow through the corresponding chambers A and B (yellow arrows), replenishing the medium in the devices. (D) Wells A2 and B2 were topped up with fresh medium (100 µL), ensuring a total of ~200 µL medium in each well.

2.3.17 Calcium imaging in primary hippocampal cultures in microfluidic devices.

Live cell Ca^{2+} imaging experiments on hippocampal cultures (12-14 DIV) in microfluidic devices were performed as previously described (Robertson *et al.*, 2014). Devices were washed three times with HBS prior to cell loading with the Ca^{2+}-sensitive dye Fluo-8 (4 µM) in HBS for 1 hour in the dark at room temperature. Following a wash step prior to experimentation (Figure 2.6), all 4 wells of the device were filled with HBS (150 µL) and 125 µL was then extracted from each of the connecting wells B1 and B2. This creates a hydrostatic

pressure gradient across the microchannels, inducing a small fluid flow from chamber A towards chamber B. Devices were then transferred to the microscope stage and cells were allowed to adapt to the hydrostatic pressure gradient for 4 minutes. Baseline recordings (4 minutes) of the cells in both chamber A and B were acquired prior to drug application. All drug solutions were added into well B1 in 50 µL increments and recorded for 4-minute periods each. Hence, vehicle (HBS) was added, followed by either glutamate (10 µM final concentration) or ADP (20 µM final concentration). At the end of each experiment, KCl (25 mM final concentration) was added to well A1 immediately followed by well B1. Ca^{2+} levels and responses from the soma of hippocampal neurons and astrocytes were recorded using an inverted microscope (Axio Observer A1, Zeiss, UK) with a 5×/0.12 objective lens (A-Plan, Zeiss, UK) and EMCCD camera (LucaR, Andor Technologies, UK) at a frame rate of 2.0 Hz and 400 ms exposure time. Time-lapse images were obtained using Andor SOLIS 4.19.30001.0 software (Andor Technologies, UK). Cells were identified as neurons and astrocytes based on their morphological characteristics and their cell specific response to ADP or high extracellular KCl. Data was analysed offline using Andor SOLIS 4.19.30001.0 software to select ROIs and gather corresponding raw data and further automated analysis was performed using MATLAB (MathWorks, R2017b) codes kindly provided by Dr. Graham Robertson. Hence, from each recording, ROIs identifying cell somas were selected in both chambers A and B and analysed separately. The corresponding mean fluorescence intensity within a ROI was computed for each time point, with the changes in fluorescence ratio expressed as $\Delta F/F0$. Multiple responses from the same ROI were included. For Ca^{2+} imaging experiments, *n* is the total number of cells analysed from at least five different devices from three independent hippocampal cultures derived from three different BALB/cJ mice.

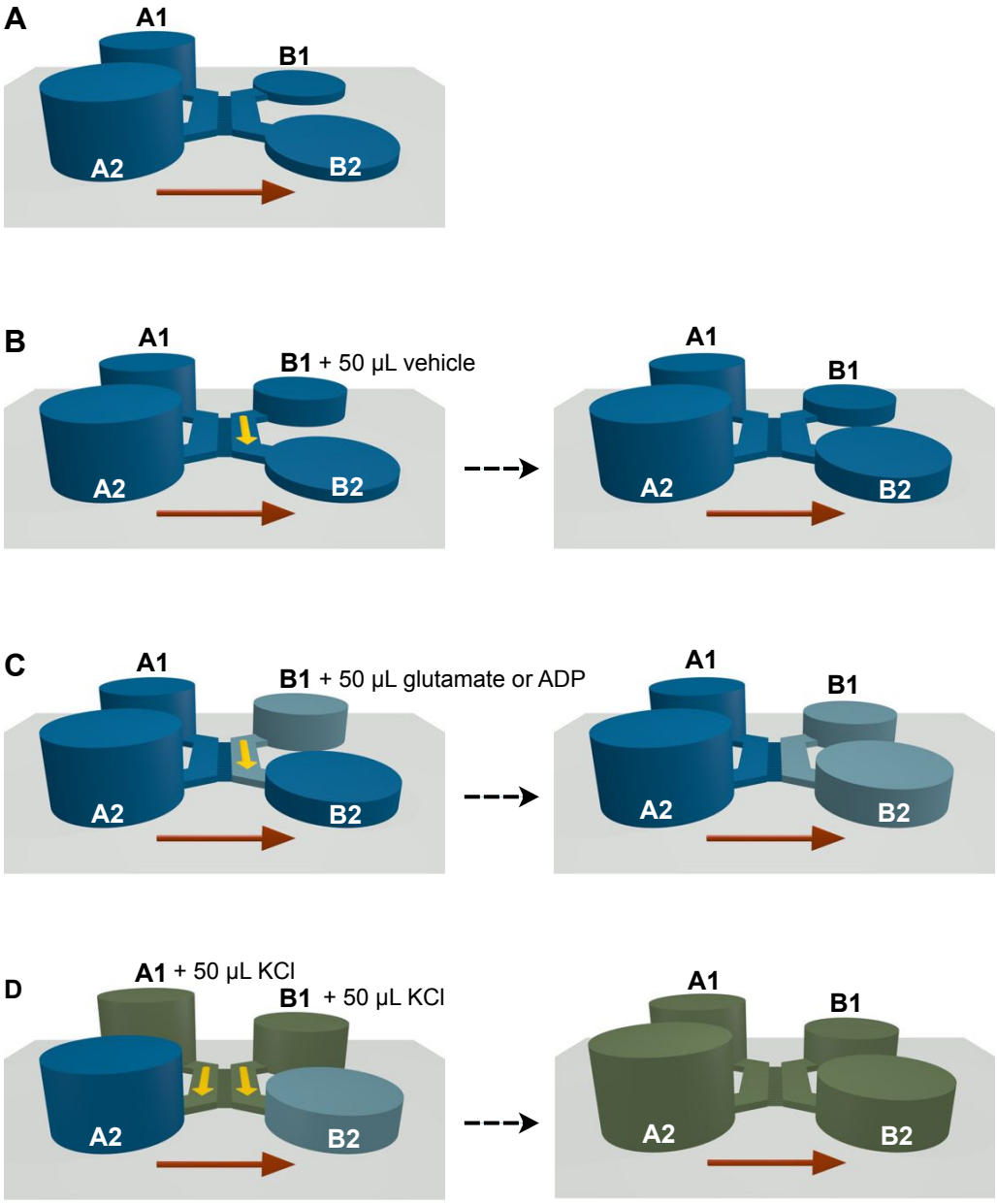

Figure 2.6. Schematic representation highlighting the experimental setup used for calcium imaging of hippocampal cells in microfluidic devices. (A) A hydrostatic pressure gradient from chamber A towards B (orange arrow) was introduced and maintained during the entire experiment. (B) 50 µL vehicle followed by (C) 50 µL glutamate or ADP was added to well B1, introducing a small fluid flow through chamber B (yellow arrow) until levels were equal in well B1 and B2 (right image). (D) 50 µL KCl was added to well A1 and B1, introducing a similar small fluid flow through chambers A and B (yellow arrows) until levels were equal in well A1 and A2 and well B1 and B2, respectively (right image).

2.3.18 Lymphocyte treatment of hippocampal cultures in microfluidic devices.

To determine the effects of lymphocytes on synaptic communication and astrocyte behaviour, live cell Ca^{2+} imaging was performed in microfluidic devices. Prior to live cell Ca^{2+} imaging, hippocampal cells obtained from BALB/cJ mice were treated (1 hour) with HCM control in chamber A and either HCM control or lymphocytes (10^8 cells mL^{-1}) in chamber B in a humidified incubator at 37 °C/5% CO_2. Vehicle (HCM) and lymphocyte cell suspensions were prepared from BALB/cJ mice (see section 2.3.12) and cell density was adjusted according to hippocampal cell number and volume loaded in the devices. Hence, medium was extracted from all 4 wells and HCM control (10 µL) was loaded into chamber A by simultaneously holding two pipette tips (5 µL each) in well A1 and A2 close to the inlet of chamber A. Subsequently, HCM control (10 µL) or lymphocyte cell suspension (10^8 cells mL^{-1}, 10 µL) was loaded into chamber B similar to vehicle loading in chamber A. The device was then placed into a humidified incubator at 37 °C/5% CO_2 for 5 minutes to ensure cell settlement, followed by the addition of HCM (50 µL) containing Fluo-8 (4 µM) in all 4 wells and put back into a humidified incubator at 37 °C/5% CO for 1 hour. Following the 1-hour treatment, cultures were washed three times with HBS and transferred onto the microscope stage, ready for imaging (see section 2.3.17).

2.3.19 Hoechst/PI staining.

To determine lymphocyte-induced neurotoxicity, hippocampal cells were stained with PI and Hoechst following lymphocyte treatment (see section 2.3.18). Hence, cultures obtained from BALB/cJ mice were washed three times with HCM and afterwards all medium was removed from all wells. PI (20 µg mL^{-1}) and Hoechst 33342 (3 µg mL^{-1}) in HCM (100 µL) was added to the wells A1 and B1 of the devices and placed into a humidified incubator at 37 °C/5% CO_2 for 30 minutes. Following staining, unbound antibodies were washed out of the devices using PBS. Hippocampal cells were imaged in PBS using an inverted epifluorescence microscope (Observer Z1, Zeiss, UK) with a 5×/0.16 Ph1 objective lens (EC Plan NeoFluar, Zeiss, UK)

and excitation using appropriate filters to visualise the fluorescent dyes. Fluorescent signals were visualized using a CMOS digital camera (ORCA-Flash 4.0, model: C11440-22CU, Hamamatsu Photonics, UK) and Zen2 imaging software (Zeiss, UK), with images analysed using Zen 2.5 (blue edition) imaging software (Zeiss, UK). All experiments were performed from three independent cultures derived from three different BALB/cJ mice.

2.3.20 Lymphocyte migration in microfluidic devices.

Devices were coated with PLO (0.01%, v/v) or collagen type I (5 μg/cm^2) for 1 hour to ensure cell adhesion. Once coated, PLO or collagen type I was washed out using phenol red-free cNB-A medium and the devices filled with phenol red-free cNB-A medium were placed into a humidified incubator at 37 °C/5% CO_2 until lymphocytes were ready to be seeded. Lymphocytes were prepared in phenol red-free cNB-A medium from BALB/cJ mice (see section 2.3.1) and loaded with the CellTracker red CMTPX dye (3 μM) for 30 minutes prior to seeding. Lymphocytes were washed once and re-suspended in phenol red-free cNB-A medium at a density of 10^8 cells mL^{-1}. Medium was then extracted from all 4 wells of the device and HCM (10 μL) and lymphocyte cell suspension (10^8 cells mL^{-1}) loading was performed as described above (see section 2.3.18). Devices containing cells were placed in a humidified incubator at 37 °C/5% CO_2 for 5 minutes ensuring cell settlement. Following this, HCM control (100 μL) was added to the lymphocytes in chamber A via well A1 and A2, and HCM control (100 μL) or the chemoattractant CCL19 (100 ng mL^{-1}) in HCM (100 μL) into the opposite chamber B via well B1 and B2. Cells were maintained in a humidified incubator at 37 °C/5% CO_2 until imaging was required. Lymphocytes were imaged at different time points (0-24 hours) using an inverted epifluorescence microscope (Observer Z1, Zeiss, UK) with a 10×/0.3 Ph1 or 20×/0.5 Ph2 objective lens (EC Plan NeoFluar, Zeiss, UK) and excitation using an appropriate filter to visualise the CellTracker red CMTPX dye. Fluorescent signals were visualized using a CMOS digital camera (ORCA-Flash 4.0, model: C11440-22CU, Hamamatsu Photonics, UK) and Zen2 imaging software (Zeiss, UK), with images

analysed using Zen 2.5 (blue edition) imaging software (Zeiss, UK). All experiments were performed from three independent cultures derived from three different BALB/cJ mice.

2.3.21 Statistical analysis.

Both experiments and analysis were not performed in a blinded manner, but experiments were randomised. All presented data are expressed as mean ± standard error of the mean (S.E.M.). *n* value refers to the number of independent lymphocyte preparations used in the case of lymphocyte experiments, while in the case of calcium imaging experiments the *n* value refers to the total number of independent cultures. Statistical significance was determined by an unpaired or paired Student's t-test, a one-way or two-way analysis of variance (ANOVA) with a Bonferroni post-hoc comparison, or a repeated one-way ANOVA with a Bonferroni post-hoc comparison where appropriate using Graphpad Prism 5.0b (GraphPad Software, Inc.), with $p<0.05$ considered to be statistically significant.

Chapter 3

Characterising lymphocyte function under primary hippocampal culture conditions.

3.1 Introduction

The CNS has long been considered an immune privileged site, with the BBB separating it from the immune system (Engelhardt, Vajkoczy and Weller, 2017). However, it is now accepted that it is not immunologically privileged and forms an essential interactive bi-directional neuroendocrine crosstalk with the immune system, guiding CNS physiological homeostasis (Blalock, 1994; Ashley and Demas, 2017). Moreover, it is now well-known that the CNS has its own immune defence system, with primarily microglia (Li and Barres, 2017) but also astrocytes (Colombo and Farina, 2016) as its central players. Furthermore, studies have shown that activated circulating T cells can cross the BBB and enter the CNS in the absence and presence of neuroinflammation (Engelhardt and Ransohoff, 2012). Recent proof of CNS antigen draining lymphatic vessels has also revived the discussion of an immunological privileged CNS (Louveau *et al*., 2015). Hence, both pathogenic and neuroprotective roles of the immune system in CNS disorders, including AD, MS and depression are currently the subject of intense investigation. In order to study cell-cell interactions in these diseases *in vitro*, co-culture systems are fundamental. This is highly dependent on the extracellular environment to fulfil its potential to investigate natural interactions between two or more cell populations without the complexity found in tissues or whole organs (Goers, Freemont and Polizzi, 2014). Therefore, this chapter highlights experimental set up considerations required for *in vitro* co-culture experiments using mouse primary hippocampal cell cultures and lymphocyte cell preparations.

While environmental control of primary cells is always important, it is even more so when two different cell types that require distinct growth media are combined in one system. Conventionally, lymphocytes are cultured in suspension in serum-containing RPMI medium, low in calcium and magnesium and high in phosphate levels (Moore, *et al*., 1967). Serum is considered a fundamental supplement in culture medium, providing essential factors, including hormones, growth factors and trace elements that promote cellular growth *in vitro*.

However, exact composition of these factors remains unknown but it is known that variation can occur between batches (Lehr, 2003), leading to unreproducible cell culturing. These unknown serum-derived substances also form a potential risk factor for microbial contamination. Antibiotics are often used to prevent microbial contamination however they can interfere with neuronal excitability via changes in ionic conductance (Bahrami and Janahmadi, 2013). Therefore, avoiding the use of serum is crucial for culturing synaptically active hippocampal cells. Furthermore, it has been shown that glial cells extensively proliferate in serum-containing medium, inhibiting neuronal growth and synaptic development (Ye and Sontheimer, 1998). In addition, excessive regression of neuronal viability and metabolic deficiency is observed using serum-containing medium (Kim *et al.*, 2013). Serums also provide intolerable amounts of glutamate, causing glutamate-induced neurotoxicity in primary cultures (Ye and Sontheimer, 1998). For neuronal cultures to grow in serum-containing medium, a high cell density is required (300 cells mm^{-2}). Considering all this, serum-free NB medium containing B27 supplement is conventionally used for long-term and low-density (down to 80 cells mm^{-2}) culturing of primary hippocampal cells (Brewer *et al.*, 1993). This medium is a modification of Dulbecco modified Eagle's medium (DMEM) and specifically designed to maintain neuronal synaptic activity without the need for an astrocyte feeding layer. Serum-free B27 supplement is an essential serum replacement and increases neuronal survival from different brain regions by more than 60%, accelerates neuronal outgrowth and improves electrophysiological activity and neuronal maturation (Brewer *et al.*, 1993; Brewer, 1995). The consequence, however, of neuronal cell growth in serum-free RPMI medium is unknown. In contrast, it has been shown that serum-free medium supports *in vitro* proliferation of human T cells (Causey *et al.*, 1994). Thus, I hypothesize that primary hippocampal cells can be co-cultured with lymphocytes in serum-free NB-A medium. Therefore, I aim to examine whether lymphocytes are viable and functional in serum-free NB-A medium, as used in our laboratory and worldwide for hippocampal cultures, using lymphocyte preparations from OT-II mice as an *in vitro* model to study lymphocyte biology in cNB-A medium.

3.2 Results

3.2.1 Lymphocyte survival is comparable in both cRPMI and cNB-A medium.

Lymphocyte survival in cRPMI medium and serum-free cNB-A medium was investigated using PI staining combined with flow cytometric analysis. Freshly isolated lymphocyte survival in cRPMI medium was 89 ± 1.4% (0 hours, n=3, Figure 3.1), which was unchanged following 1 hour of culturing (91.2 ± 1.8%, n=3, Figure 3.1), but decreased significantly following 18 hours (65.1 ± 2.3%, n=3, $p<0.001$ vs 0 hours, Figure 3.1), 48 hours (28.1 ± 4.8%, n=3, $p<0.001$ vs 0 hours, Figure 3.1) and 72 hours (12.8 ± 1.5%, n=3, $p<0.001$ vs 0 hours, Figure 3.1). In comparison, freshly isolated lymphocyte survival in cNB-A medium was 87.1 ± 2.3% (n=3, Figure 3.1) and 84 ± 2.6% following 1 hour in culture (n=3, Figure 3.1), which significantly decreased in a similar manner following 18 hours (61.9 ± 0.6%, n=3, $p<0.001$ vs 0 hours, Figure 3.1), 48 hours (25.8 ± 1.9%, n=3, $p<0.001$ vs 0 hours, Figure 3.1) and 72 hours (10.5 ± 0.6%, n=3, $p<0.001$ vs 0 hours, Figure 3.1). Hence, no significant difference was observed between lymphocyte survival in conventional cRPMI and cNB-A medium.

3.2.2 Cell types in lymphocyte preparations are unchanged in cNB-A medium.

Having established that both media are equally sufficient in maintaining cell survival, I assessed whether cNB-A medium affects the composition and ratio of lymphocyte subsets at 18 hours *in vitro*. Flow cytometric analysis showed significantly reduced $CD45.2^+$ leukocyte numbers when cultured in cNB-A medium (95.6 ± 0.4%, n=3, $p<0.01$ vs cRPMI, Figure 3.2A and B) compared to cRPMI medium (99.2 ± 0.1%, n=3, Figure 3.2A and B). However, no change in both B cells ($CD3^-B220^+$, cRPMI: 42.9 ± 1.9%, cNB-A: 41.5 ± 1.5%, n=3, Figure 3.2A, C and D) and CD3 T cells ($CD3^+B220^-$, cRPMI: 51.7 ± 1.7%, cNB-A: 49.1 ± 1.9%, n=3, Figure 3.2A, C and D) was observed when comparing both media types. Furthermore, CD4 Th cell ($CD3^+CD4^+$) and cytotoxic CD8 T cell ($CD3^+CD4^-$) numbers when cultured in cNB-A medium (CD4: 42.5 ± 1.7%, CD8: 6.6 ± 0.4%, n=3, Figure 3.2A, E and F) were not significantly different from those observed in cRPMI medium (CD4: 44.3 ± 1.4%, CD8: 7.4 ±

0.5%, n=3, Figure 3.2A, E and F). Despite the small but significant reduction in CD45.2^{+} leukocyte numbers, B to T cell ratio (1:1) was unaltered in both media tested, with a 6:1 ratio of CD4 to CD8 T cells in both media, as previously reported (Barnden *et al.*, 1998).

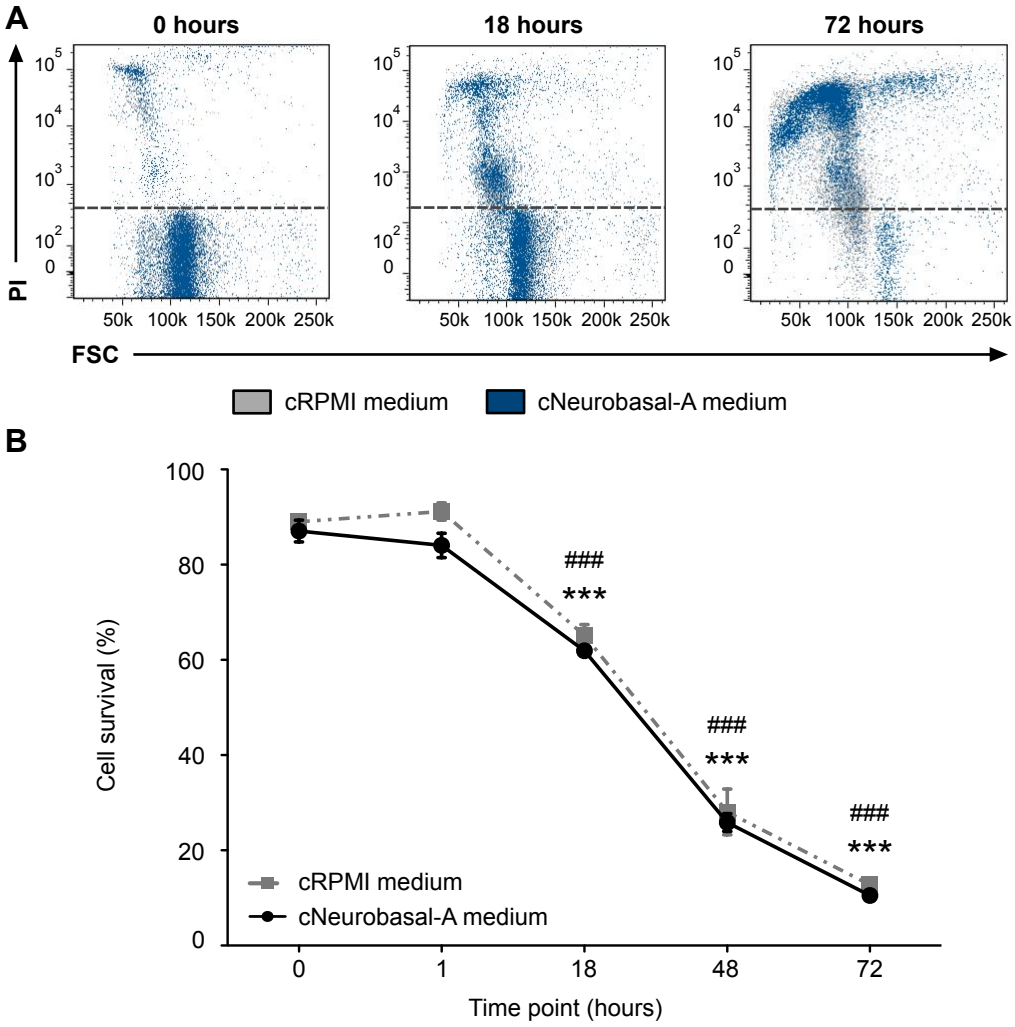

Figure 3.1. Lymphocyte survival is unchanged in cNB-A media when compared to conventional cRPMI. (A) Representative dot-plots showing PI^{+} and PI^{-} lymphocytes cultured in cRPMI and cNB-A medium. (B) Time course revealing cell survival (percentage of PI^{-} lymphocyte) in cNB-A medium is similar to cRPMI. Data are presented as mean ± S.E.M. and analysed using a two-way ANOVA with a Bonferroni post-hoc comparison. *** = $p<0.001$ vs 0 hours, cRPMI medium and ### = $p<0.001$ vs 0 hours, cNB-A medium. All experiments were carried out in triplicate from three separate OT-II lymphocyte preparations (n=3).

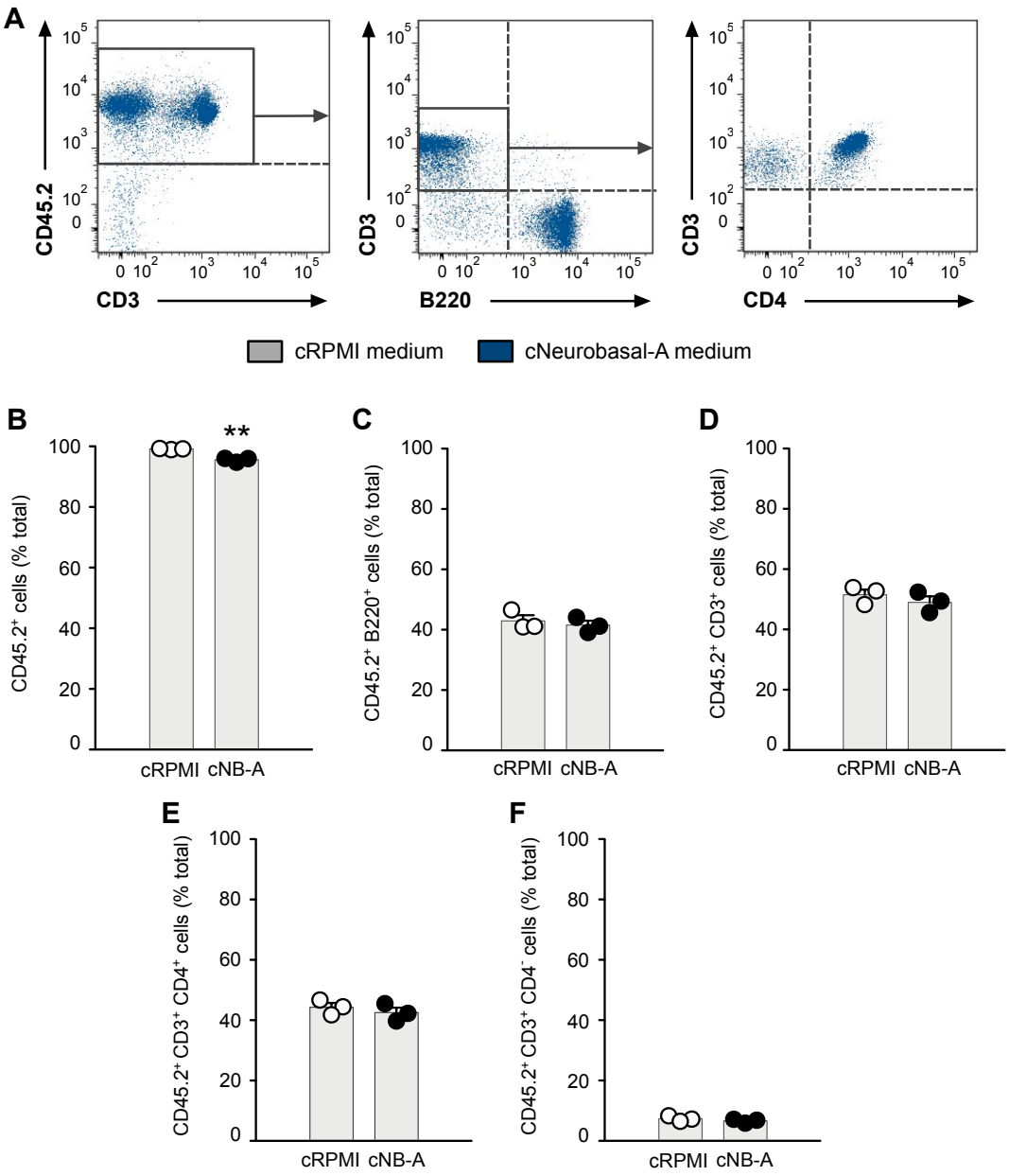

Figure 3.2. Lymphocyte preparation cell type composition is unaffected in cNB-A medium. (A) Representative dot-plots showing CD45.2, B220, CD3 or CD4 expressing lymphocytes cultured in cRPMI and cNB-A medium. Bar charts with scatter plots highlighting the effect of cNB-A medium on (B) CD45.2 leukocytes, (C) B cells, (D) T cells, (E) CD4 Th cells and (F) cytotoxic CD8 T cells. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t test for each cell marker. ** = $p<0.01$ vs cRPMI medium. All experiments were carried out in triplicate from three separate OT-II lymphocyte preparations (n=3).

3.2.3 T cell proliferation and cytokine release is maintained in cNB-A medium.

Having confirmed that both lymphocyte survival and subsets are unaltered in cNB-A medium, I examined whether lymphocyte proliferation in response to the ovalbumin$_{323-339}$ peptide was affected in cNB-A medium. Flow cytometric analysis showed significantly increased numbers of proliferating T cells ($CD4^+Ki\text{-}67^+$) in both cRPMI (26.0 ± 2.6%, n=4, $p<0.001$ vs control, Figure 3.3) and cNB-A medium (29.5 ± 3.3%, n=4, $p<0.001$ vs control, Figure 3.3) in response to the ovalbumin$_{323-339}$ peptide when compared to non-stimulated control T cells (cRPMI: 5.1 ± 2.0%, cNB-A: 3.6 ± 0.6%, n=4, Figure 3.3). Hence, the ability of $CD4^+$ Th cells to proliferate was comparable between the two media types.

Having confirmed that $CD4^+$ Th cell proliferation is unchanged in cNB-A medium, I investigated whether ovalbumin$_{323-339}$ peptide stimulation-induced cytokine release also remained viable. Stimulation-induced IFN-γ release was significantly upregulated in both cRPMI (21.7 ± 4.9 ng mL^{-1}, $p<0.01$ vs control, n=4, Figure 3.4A) and cNB-A medium (27.8 ± 4.7 ng mL^{-1}, $p<0.001$ vs control, n=4, Figure 3.4A) when compared to non-stimulated controls (cRPMI: 0.7 ± 0.7 ng mL^{-1}, cNB-A: 1.3 ± 0.6 ng mL^{-1}, n=4, Figure 3.4A). In contrast, IL-5 release was not significantly increased by ovalbumin$_{323-339}$ peptide-stimulated $CD4^+$ Th cells in both cRPMI (283.5 ± 132.2 ng mL^{-1}, n=4, Figure 3.4B) and cNB-A medium (121.5 ± 91.5 ng mL^{-1}, n=4, Figure 3.4B) when compared to non-stimulated controls (cRPMI: 122.4 ± 67.7 pg mL^{-1}, cNB-A: 95.3 ± 51.3 pg mL^{-1}, n=4, Figure 3.4B). Analogous to $CD4^+$ Th cell proliferation, no significant difference in ovalbumin$_{323-339}$ peptide stimulation-induced IFN-γ and IL-5 release was observed between the two media tested.

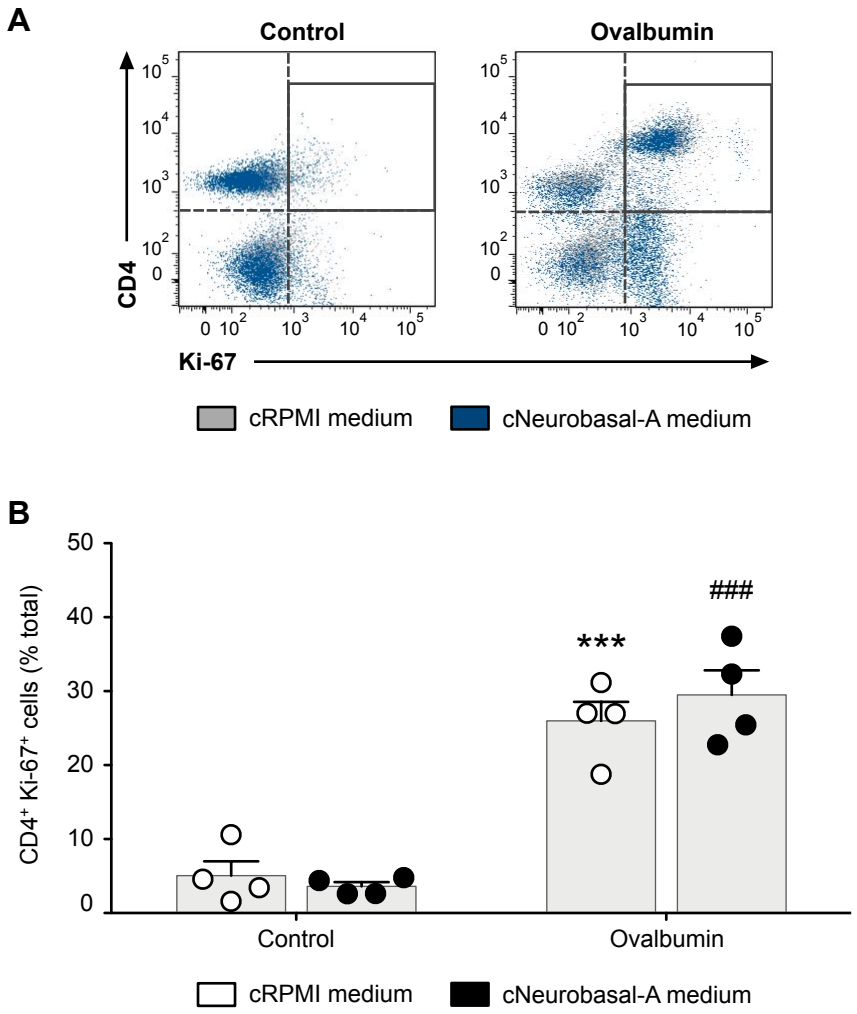

Figure 3.3. CD4$^+$ Th cell proliferation is unaltered in cNB-A media. (A) Representative dot-plots showing CD4$^+$Ki-67$^+$ lymphocytes cultured in cRPMI and cNB-A medium under non-stimulated (control) and ovalbumin$_{323-339}$ peptide-stimulated (ovalbumin) conditions. (B) Bar chart with scatter plots revealing CD4$^+$ Th cell proliferation is unchanged following ovalbumin-stimulation when cultured in cNB-A medium. Data are presented as mean ± S.E.M. and analysed using a two-way ANOVA with a Bonferroni post-hoc comparison. *** = $p<0.001$ vs control cRPMI and ### = $p<0.001$ vs control cNB-A medium. All experiments were carried out in triplicate from four separate OT-II lymphocyte preparations (n=4).

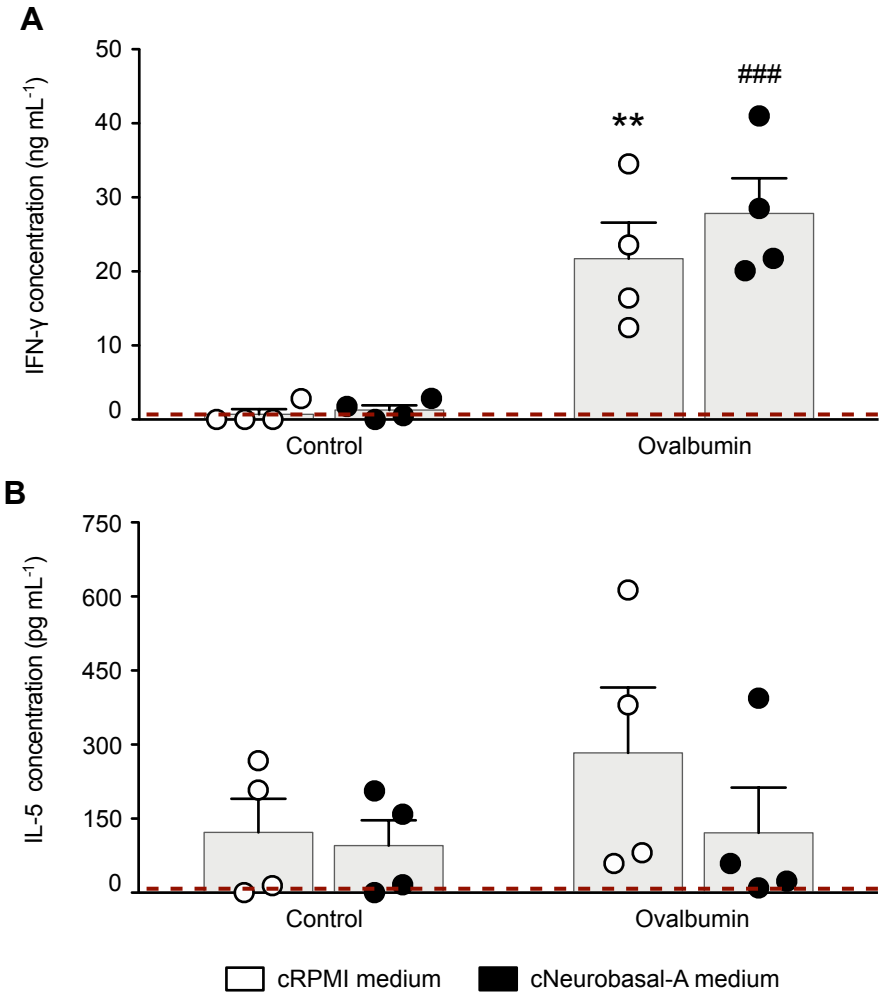

Figure 3.4. Cytokine release is comparable in cNB-A medium vs conventional cRPMI medium. (A) Bar chart with scatter plots summarising no effects of cNB-A vs cRPMI medium on non-stimulated (control) and ovalbumin$_{323-339}$ peptide stimulation-induced release of IFN-γ and (B) IL-5. Data are presented as mean ± S.E.M. and analysed using a two-way ANOVA with a Bonferroni post-hoc comparison. ** = $p<0.01$ vs control cRPMI, and ### = $p<0.001$ vs control cNB-A medium. All experiments were carried out in triplicate from four separate OT-II lymphocyte preparations ($5x10^6$ cells mL^{-1}, n=4). Red dotted line indicates detection limit.

As previously established by our laboratory, IL-16 present in conditioned medium from lymphocytes (LCM) is neuroprotective against KA-induced toxicity and OGD-induced cell death (Shrestha et al., 2014). Therefore, I investigated whether IL-16 is spontaneously released by primary lymphocytes in cRPMI medium as well as in cNB-A medium. In agreement with previous finding, IL-16 was spontaneously released by non-stimulated controls in both media (cRPMI: 918.1 ± 121.8 pg mL^{-1}, cNB-A: 763.9 ± 107.5 pg mL^{-1}, n=4, Figure 3.5). In contrast to $ovalbumin_{323-339}$ peptide stimulation-induced IFN-γ release, IL-16 release was not significantly increased following $ovalbumin_{323-339}$ peptide stimulation of $CD4^+$ Th cells in either cRPMI (756.6 ± 87.3 pg mL^{-1}, n=4, Figure 3.5) or cNB-A medium (833.9 ± 150.2 pg mL^{-1}, n=4, Figure 3.5) when compared to non-stimulated controls. Hence, no significant difference in stimulation-induced IL-16 release was observed between the two different media types tested.

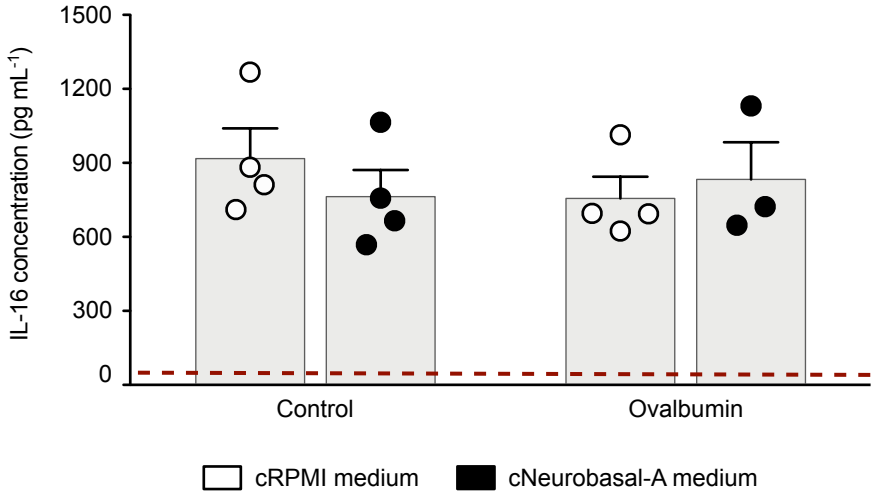

Figure 3.5. Spontaneous IL-16 release is similar in cNB-A medium. Bar chart with scatter plots summarising IL-16 release of non-stimulated control cells and $ovalbumin_{323-339}$ peptide stimulated cells in cNB-A vs cRPMI medium. Data are presented as mean ± S.E.M. and analysed using a two-way ANOVA with a Bonferroni post-hoc comparison. All experiments were carried out in triplicate from four separate OT-II lymphocyte preparations ($5x10^6$ cells mL^{-1}, n=4). Red dotted line indicates detection limit.

3.2.4 IL-16 is constitutively released by various lymphocyte cell types.

Having confirmed that lymphocyte functionality is maintained and IL-16 is constitutively released under primary hippocampal culture conditions using cNeurobasal-A medium, I examined by which cell type IL-16 is released. Hence, $CD4^+$ Th cells were isolated, creating an enriched $CD4^+$ Th and $CD4^+$ depleted cell suspension (see section 2.3.4), with both suspensions cultured under non-stimulated conditions for 72 hours (see section 2.3.6). $CD4^+$ Th cells accounted for 89.5 ± 1.8% of the enriched $CD4^+$ Th cell suspension (n=3, $p<0.001$ vs control, Figure 3.6), which was significantly higher than the non-isolated control cell suspension (27.5 ± 2.7%, n=3, Figure 3.6). As expected, the $CD4^+$ depleted cell suspension had significant fewer $CD4^+$ Th cells (4.8 ± 0.9%, n=3, $p<0.001$ vs control and $CD4^+$, Figure 3.6) compared to both the non-isolated control and the enriched $CD4^+$ Th cell suspension.

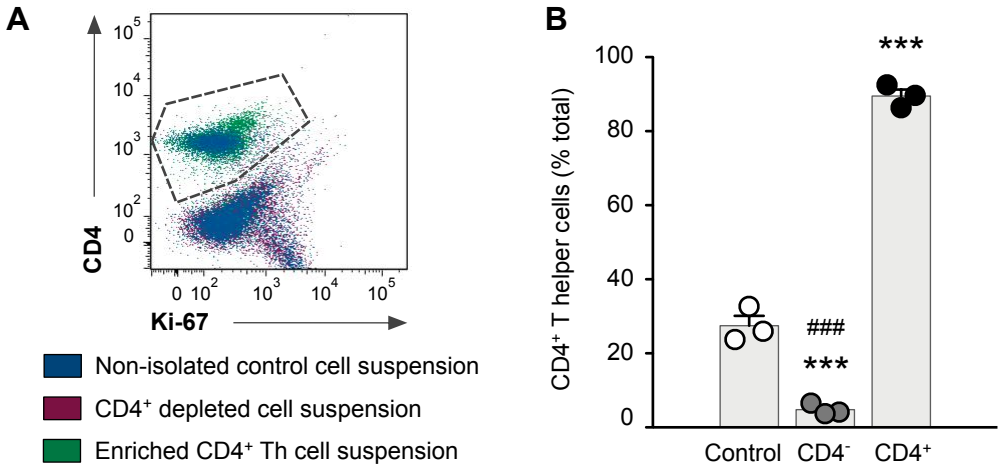

Figure 3.6. $CD4^+$ Th cells account for ~90% of the enriched $CD4^+$ Th cell suspension. (A) Representative dot-plot showing $CD4^+$ Th cells in non-isolated control, $CD4^+$ depleted and enriched $CD4^+$ Th cell suspensions. (B) Bar chart with scatter plots summarising increased $CD4^+$ Th cell percentage in enriched $CD4^+$ Th cell suspensions ($CD4^+$), whereas this is decreased in $CD4^+$ depleted cell suspensions ($CD4^-$). Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. *** = $p<0.001$ vs control and ### = $p<0.001$ vs $CD4^+$. All experiments were carried out in triplicate from four separate OT-II lymphocyte preparations (n=4).

Having confirmed $CD4^+$ Th cell isolation, I examined whether IL-16 is spontaneously released by non-stimulated $CD4^+$ Th cells in the enriched $CD4^+$ Th cell suspension, as I previously demonstrated that IL-16 release from non-isolated control cell suspensions is spontaneous (see Figure 3.5). IL-16 levels were significantly higher in the enriched $CD4^+$ Th cell suspension (663.9 ± 89.8 pg mL^{-1}, n=3, $p<0.05$ vs control and $p<0.01$ vs $CD4^-$, Figure 3.7) compared to both the non-isolated control (232.4 ± 47.2 pg mL^{-1}, n=3, Figure 3.7) and $CD4^+$ depleted cell suspension (167.5 ± 61 pg mL^{-1}, n=3, Figure 3.7).

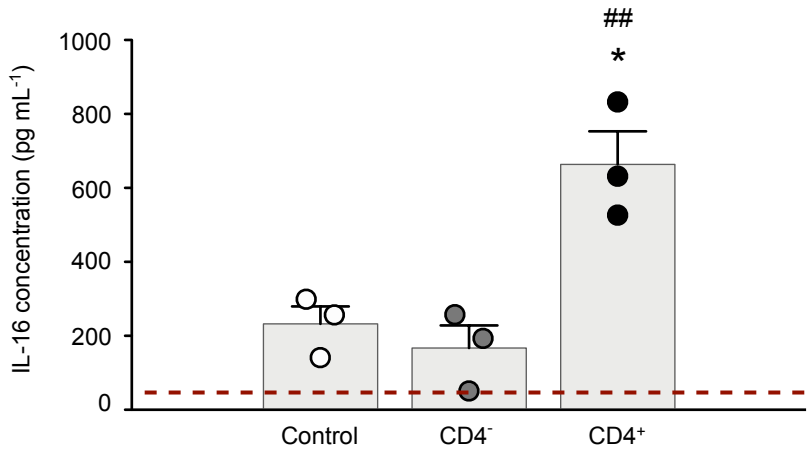

Figure 3.7. IL-16 is spontaneously released in all three cell suspensions. Bar chart with scatter plots summarising increased IL-16 levels present in non-stimulated enriched $CD4^+$ Th cell suspensions ($CD4^+$). Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. * = $p<0.05$ vs control and ## = $p<0.01$ vs $CD4^-$. All experiments were carried out in triplicate from four separate OT-II lymphocyte preparations ($2.5x10^6$ cells mL^{-1}, n=4). Red dotted line indicates detection limit.

3.2.5 IL-16 release from various lymphocyte cell types is ovalbumin$_{323-339}$ peptide stimulation-independent.

Having shown that IL-16 is constitutively released by naïve isolated $CD4^+$ Th cells, comparable to previously presented data regarding naïve non-isolated control suspensions (see Figure 3.5), I investigated whether IL-16 release by isolated $CD4^+$ Th cells is increased under

ovalbumin$_{323-339}$ peptide-stimulated conditions. As the enriched CD4$^+$ Th cell suspension exists for ~90% of CD4$^+$ Th cells (Figure 3.6) and therefore minimal APCs are isolated in this cell suspension, BMDCs were added to each cell suspension to aid in presenting the ovalbumin$_{323-339}$ peptide to CD4$^+$ Th cells. First, I confirmed that isolated CD4$^+$ Th cells were stimulated by examining the ability of isolated CD4$^+$ Th cells to release ovalbumin$_{323-339}$ peptide stimulation-induced cytokines. Stimulation-induced IFN-γ levels in both the non-isolated control (7.3 ± 1.1 ng mL^{-1}, n=3, $p<0.01$ vs non-stimulated control, Figure 3.8A) and enriched CD4$^+$ Th cell suspension (16.4 ± 2.0 ng mL^{-1}, n=3, $p<0.001$ vs non-stimulated CD4$^+$, Figure 3.8A) but not in the CD4$^+$ depleted cell suspension (4.8 ± 2.2 ng mL^{-1}, n=3, Figure 3.8A) were significantly increased when compared to non-stimulated cell suspensions (control: 0 ± 0 ng mL^{-1}, CD4$^+$: 0 ± 0 ng mL^{-1}, CD4$^-$: 0 ± 0 ng mL^{-1}, n=3, Figure 3.8A). Furthermore, other stimulation-induced cytokine markers, including IL-2 and IL-17α were increased following ovalbumin$_{323-339}$ peptide stimulation in all three cell suspensions compared to non-stimulated cells (n=1, Figure 3.8B & C).

In agreement with previous presented data regarding IL-16 release by non-stimulated and stimulated control cell suspensions (see Figure 3.5), all three cell suspensions showed no significant difference in IL-16 levels released by non-stimulated cells (control: 315.4 ± 55.8 pg mL^{-1}, CD4$^-$: 249.6 ± 32.8 pg mL^{-1}, CD4$^+$: 746.1 ± 110.7 pg mL^{-1}, n=3, Figure 3.9) compared to ovalbumin$_{323-339}$ peptide-stimulated cells (control: 370.9 ± 63.6 pg mL^{-1}, CD4$^-$: 365.6 ± 15.2 pg mL^{-1}, CD4$^+$: 508 ± 135.1 pg mL^{-1}, n=3, Figure 3.9). Furthermore, BMDCs alone did not release IL-16 under non-stimulated nor ovalbumin$_{323-339}$ peptide-stimulated conditions (Figure 3.9).

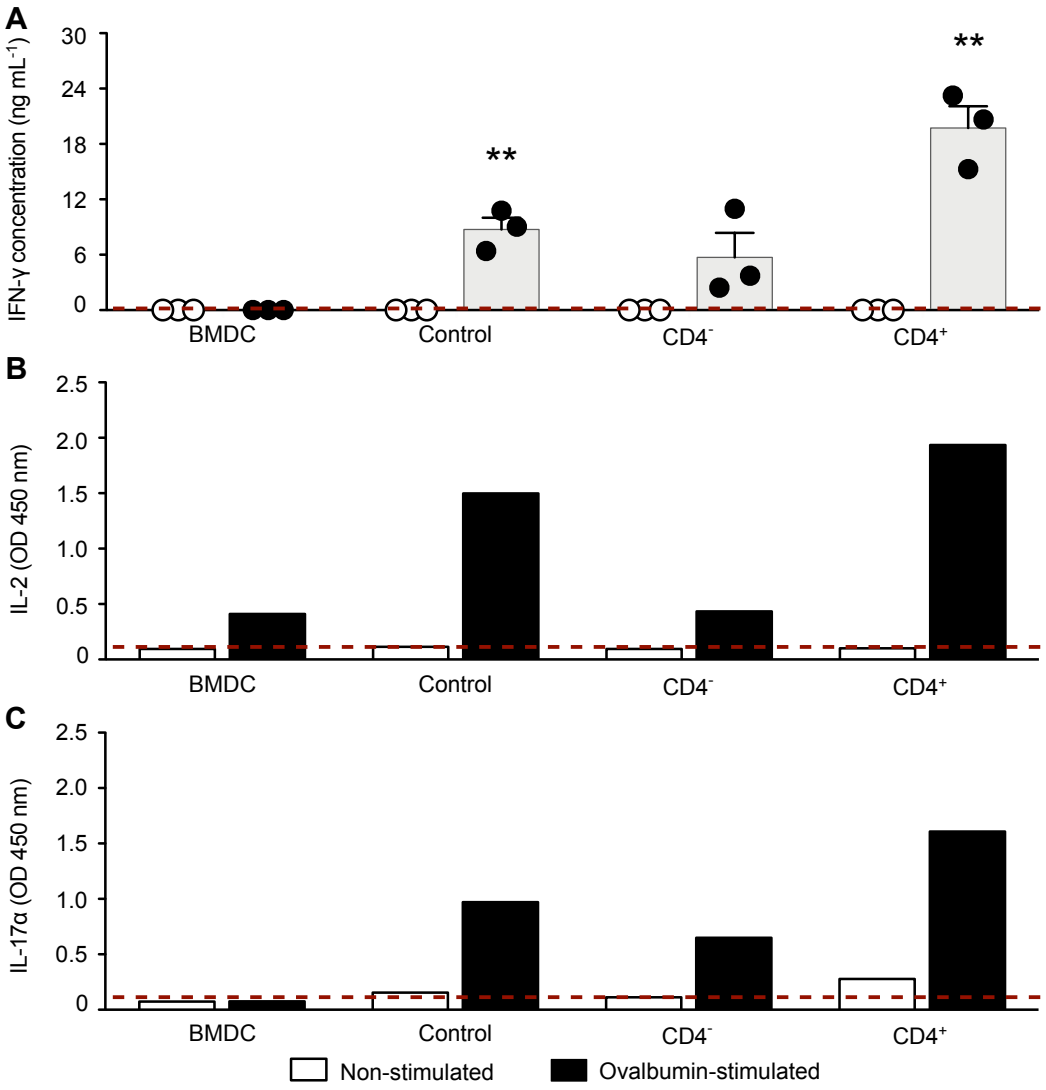

Figure 3.8. Ovalbumin$_{323-339}$ peptide stimulation induces cytokine release from isolated and control cell suspensions. (A) Bar chart with scatter plots summarizing stimulation-induced increases in IFN-γ in non-isolated control and enriched $CD4^+$ Th cell suspensions ($CD4^+$), but not in BDMC and $CD4^+$ depleted cell suspension ($CD4^-$). (B & C) Bar chart illustrating ovalbumin stimulation-induced increases in IL-2 and IL-17α from non-isolated control (BMDC and control), $CD4^+$ depleted ($CD4^-$) and enriched $CD4^+$ Th cell suspensions ($CD4^+$). Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t test. ** = $p<0.01$ vs non-stimulated cells. Experiments were carried out in triplicate from (A) three separate OT-II lymphocyte preparations ($2.5x10^6$ cells mL^{-1}, n=3) and (B & C) one OT-II lymphocyte preparation ($2.5x10^6$ cells mL^{-1}, n=1). Red dotted line indicates detection limit.

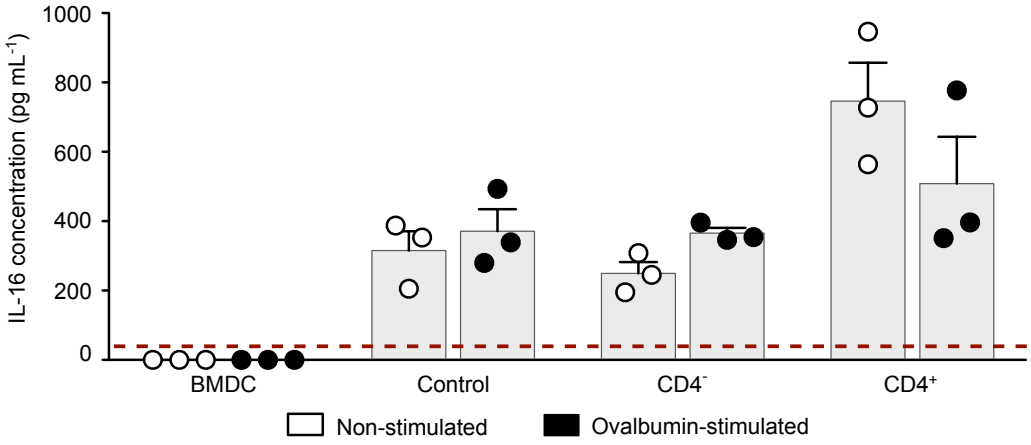

Figure 3.9. IL-16 release is independent of ovalbumin$_{323-339}$ peptide stimulation in all three cell suspensions. Bar chart with scatter plots summarising no difference between IL-16 release from naïve and ovalbumin$_{323-339}$ peptide-stimulated BMDCs, non-isolated controls, $CD4^+$ depleted ($CD4^-$) or enriched $CD4^+$ Th cell suspensions ($CD4^+$). Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t test. All experiments were carried out in triplicate from three separate OT-II lymphocyte preparations ($2.5x10^6$ cells mL^{-1}, n=3). Red dotted line indicates detection limit.

3.3 Discussion

In order to study hippocampal-lymphocyte cellular interactions, I investigated the experimental set up, including the extracellular environment required for *in vitro* co-culture experiments. OT-II mice (backcrossed on a C57BL/6J background) are widely used to study T cell biology, including T cell activation. The α- and β-chain TCR of their $CD4^+$ Th cells recognizes the $ovalbumin_{323-339}$ peptide when presented by MHC II molecules on APCs (Barnden *et al.*, 1998). This will then induce T cell proliferation and activation. As such, immune responses can easily be studied by direct administration of the $ovalbumin_{323-339}$ peptide to primary lymphocyte cell preparations, including APCs such as B cells and DCs, obtained from these mice. Therefore, OT-II lymphocytes are used in this chapter as a proof-of-principle model to assess lymphocyte viability and functionality in serum-free NB-A medium.

3.3.1 Primary lymphocytes survive and are functional in the same medium as primary hippocampal cells.

In this chapter, I have demonstrated that cNB-A medium, as is widely used for mouse and rat primary hippocampal cultures, maintains lymphocyte viability and functionality. Initial experiments were conducted to verify lymphocyte viability in cNB-A medium. Hence, collected data revealed that lymphocyte viability at different time points in cNB-A medium was similar and not significantly different from lymphocyte viability in conventional cRPMI medium. In agreement with previous experiments in our laboratory (Shrestha 2011; Thesis), cell viability in both media was approximately 60% at 18 hours *in vitro*. In contrast to cell viability, a small but significant difference in $CD45.2^+$ leukocyte numbers was observed between conventional cRPMI and cNB-A medium at this time point. Considering no reasonable explanation for the difference in general leukocyte numbers and giving the low error bars, it is not recognized as a relevant significant difference and cNB-A medium is accepted to be sufficient in maintaining lymphocyte cell type composition. The CD45.2 antigen is known to be expressed on all nucleated hematopoietic cells (Scheid and Triglia,

1979) and therefore accepted as a general leukocyte marker. This CD45 transmembrane protein-tyrosine phosphatase exists as multiple isoforms, generated via alternative splicing of exons 4-6 and are cell type- and activation-dependent (Tong, *et al*., 2005). These different isoforms might explain the difference in $CD45.2^+$ leukocyte numbers when cultured in cNB-A medium, however, as these isotypes are cell type-dependent (Tong *et al*., 2005) and no differences were observed in lymphocyte cell types as well as in the subset ratio when comparing the two media tested, this is rather unlikely. Furthermore, even though both media tested differ in their composition (serum vs B27 supplement), differences described do not necessarily explain the significant change found in $CD45.2^+$ leukocyte numbers. In fact, the few extra factors present in NB-A medium, including L-alanine, ferric nitrate, zinc sulphate and HEPES have been suggested to be essential for lymphocyte cell growth and maturation, and provide excellent buffering capacities (Darzynkiewicz and Jacobson, 1971; Nordlind, *et al*., 1979; Carpentieri *et al.*, 1986). In addition, while the B27 supplement has been developed especially for neurons, all components, including vitamins, essential fatty acids, hormones, and anti-oxidants (Brewer *et al*., 1993) are considered beneficial for the growth and maturation of any cell type.

Both serum-containing cRPMI and serum-free cNB-A medium sufficiently provided the right conditions for $CD4^+$ Th cell proliferation, which is in contrast to previous studies showing that serum is beneficial for differentiation and proliferation (Brunner *et al.*, 2010). However, it has also been reported that lymphocytes cultured in serum-free medium proliferate sufficiently and perform their effector functions, including cytokine release comparable to those cultured in serum-containing medium (Causey *et al.*, 1994). In agreement with this aforementioned study, I have shown that ovalbumin$_{323-339}$ peptide stimulation-induced IFN-γ release was not affected by serum-free cNB-A medium. Stimulation-induced IL-5 release, however, was not increased in either media compared to non-stimulated controls. This is in accordance with other studies, where it is suggested that OT-II T cells produce a Th1-type cytokine (IFN-γ)

profile with significantly reduced Th2 (IL-5) responses (Leung *et al.*, 2013). Next to assessing IFN-γ and IL-5 release in cNB-A medium, I determined IL-16 release as our laboratory has identified this cytokine as the underlying mechanism that induces lymphocyte-mediated neuroprotection following KA- or OGD-induced neurotoxicity. To further explore this underlying mechanism in this study, it had to be confirmed first whether lymphocytes release IL-16 under my experimental conditions. In agreement with our previous study (Shrestha *et al.*, 2014), IL-16 was constitutively released by lymphocytes cultured in serum-containing cRPMI as well as serum-free cNB-A medium. This finding is also confirmed in other studies, where cytotoxic $CD8^+$ T cells (Wu *et al.*, 1999), monocyte-derived DCs (Kaser *et al.*, 1999) and B cells (Kaser *et al.*, 2000) constitutively release IL-16. This constitutive secretion mechanism is also observed for other cytokines, including TNF-α release from macrophages (Lieu *et al.*, 2008). To the best of my knowledge, this is the first study looking into IL-16 release from ovalbumin-stimulated lymphocytes, as other studies have only shown IL-16 release from histamine-stimulated cytotoxic $CD8^+$ T cells (Laberge *et al.*, 1995), concanavalin-A-stimulated cytotoxic $CD8^+$ T cells (Zhang *et al.*, 1998) or CD3/CD28-stimulated cytotoxic $CD8^+$ T cells and $CD4^+$ Th cells (Wu *et al*., 1999).

3.3.2 IL-16 is constitutively released by various lymphocyte cell types.

Having shown that IL-16 was constitutively released by lymphocyte preparations, I examined which lymphocyte cell type was responsible for IL-16 release used under my experimental conditions. IL-16 is known to be synthesized by a variety of immune and non-immune cells, including cytotoxic $CD8^+$ T cells and $CD4^+$ Th cells (Center *et al*., 1982), monocytes (Elssner *et al*., 2004), eosinophils and mast cells (Laberge *et al*., 1999), epithelial cells (Cheng *et al*., 2001), DCs (Kaser *et al*., 1999), B cells (Sharma *et al*., 2000), microglia (Guo *et al*., 2004) and neurons (Kurschner *et al*., 1999). Data generated in this chapter suggests that IL-16 is produced and released by $CD4^+$ Th cells in spleen and lymph node cell preparations, as a significantly higher concentration of IL-16 is observed in the enriched $CD4^+$ Th cell suspension compared

to the control and $CD4^+$ depleted cell suspension. However, this data needs to be interpreted carefully, considering there is no significant difference between $CD4^+$ depleted and control cell suspensions. If $CD4^+$ Th cells were the only cells releasing IL-16 in all three cell suspensions investigated and one were to normalise for the absolute $CD4^+$ Th cell number, IL-16 concentration in the $CD4^+$ depleted cell suspension was expected to be lower. Thus, whether $CD4^+$ Th cells in spleen and lymph node preparations are the sole producer of IL-16 cannot be concluded from this data. It can be implied though that $CD4^+$ Th cells, but also other cells from spleen and lymph node preparations, do indeed release IL-16. Indeed, monocyte-derived DCs have been shown to constitutively release IL-16 at 6 DIV (Kaser *et al*., 1999). However, upon full maturation (after 6 DIV), no IL-16 activity as well as mRNA levels are found within monocyte-derived DCs (Kaser *et al*., 1999), which agrees with BMDCs in my experiments not releasing IL-16 irrespective of their activation state. Other studies have shown that B-cells constitutively express IL-16 mRNA and secrete IL-16 (Kaser *et al*., 2000, Sharma *et al*., 2000). However, IL-16 release from highly purified B cell preparations was not investigated due to time limitations. The same accounts for IL-16 release from highly purified cytotoxic $CD8^+$ T cell preparations. These cells are known to constitutively express and release IL-16, as they constitutively contain active caspase-3, irrespective of cellular apoptosis, which is responsible for cellular IL-16 release (Zhang *et al*., 1998, Wu *et al*., 1999). This particular study also showed that even though pro-IL-16 mRNA is constitutively expressed in $CD4^+$ Th cells, its release is only achieved following antigenic stimulation (Wu *et al*., 1999), which contradicts data presented in this chapter, as the enriched $CD4^+$ Th cell suspension revealed high levels of IL-16 under both non-stimulated and ovalbumin-stimulated conditions. Therefore, further experiments would be required to fully confirm that IL-16 release in all three cell suspensions is from $CD4^+$ Th cells, especially because the enriched $CD4^+$ Th cell suspension consists for 90% of $CD4^+$ Th cells. It could be that the other 10% contains other lymphocyte cell types, but it is more likely that cells start to lose their CD4 receptor the longer they are in culture. To confirm that the enriched $CD4^+$ Th cell suspension consists purely out of $CD4^+$ Th cells, flow

cytometric analysis could be performed to stain for other lymphocyte cell types, including B cells, cytotoxic $CD8^+$ T cells and DCs. Furthermore, given that the release of bioactive IL-16 from $CD4^+$ Th cells is correlated with pro-caspase-3 cleavage into active caspase-3 (Zhang *et al.*, 1998), flow cytometric analysis or western blots could be performed to confirm active caspase-3 in unstimulated $CD4^+$ Th cells under my experimental conditions. This could indicate whether unstimulated $CD4^+$ Th cells are capable of secreting IL-16.

In conclusion, data in this chapter shows that lymphocytes are viable and fully functional in medium that is required for the sufficient growth of primary hippocampal cell cultures. Therefore, co-culture experiments can be conducted in cNB-A medium supplemented with serum-free B27 in order to study contact-dependent and IL-16-induced contact-independent effects of lymphocytes on hippocampal cellular activity.

Chapter 4

Determining contact-independent effects of lymphocytes on pharmacologically-induced intracellular calcium increases in hippocampal cultures.

4.1 Introduction

Most CNS disorders, including AD, MS and stroke are considered complex diseases with multifactorial aetiologies, with inflammation recognized as a central element. Hence, it is not only been thought that inflammation induces neurodegeneration (Hosseini *et al.*, 2018) it is also suggested that neurodegeneration initiates inflammatory processes, thus worsening symptoms and contributing to disease progression (Wang *et al.*, 2018). However, innate (Komine *et al.*, 2018) and adaptive immune responses (Neal *et al.*, 2018) have also been shown to be neuroprotective. Whilst many attempts have been made to understand the inflammatory mechanisms involved in CNS development, homeostasis and disorders, it is clear that many questions still remain. Therefore, this chapter has made a beginning in better understanding the role of the inflammatory mediator IL-16 on neuronal and astrocytic function.

Cytokines are inflammatory mediators that play a substantial role in the body's defense system and are categorized as either pro- or anti-inflammatory. Within the CNS, these neuromodulatory factors are not only released by infiltrating peripheral immune cells, but recent evidence has shown that parenchymal cells, including neurons (Lim *et al.*, 2016), astrocytes (Choi *et al.*, 2014), microglia (vom Berg *et al.*, 2012), oligodendrocytes (Gadani *et al.*, 2015) and microvascular endothelial cells (O'Carroll *et al.*, 2015) also produce and release cytokines. By binding to their cognate receptors, they form a complex autocrine and paracrine cross-talk between multiple cell types in the CNS and aid in physiological functions, including neurite outgrowth, neurogenesis, synaptic development and synaptic plasticity (Vezzani and Viviani, 2015; de Miranda *et al.*, 2017; Levin and Godukhin, 2017). In addition, elevated levels of inflammatory cytokines are observed in most neuroinflammatory disease states, including AD, MS and PD (Martins *et al.*, 2011; Garcia-Esparcia *et al.*, 2014; Zheng, Zhou and Wang, 2016). Cytokines known to interfere with CNS functioning are the well-known pro-inflammatory cytokines IL-1β, IL-6 and IFN-β (Allan and Rothwell, 2001). Indeed, IL-1β enhances NMDA-induced intracellular Ca^{2+} $[Ca^{2+}]_i$ increases in rat hippocampal neurons,

mediated by the activation of Src tyrosine kinase and might contribute to glutamate-mediated neurodegeneration (Viviani *et al.*, 2003). Furthermore, chronic IL-6 exposure to primary CPNs reduces action potential generation, increases input resistance, and enhances the electrical response to AMPA as well as increases AMPA-induced increases in $[Ca^{2+}]_i$ (Nelson, Ur and Gruol, 2002). In addition, IFN-β1a reduces striatal excitatory post-synaptic currents amplitude, which is mediated by Ca^{2+}, CaMKII and the GluN2A-containing NMDA receptors (Di Filippo *et al.*, 2016).

In addition to these classical cytokines, others have been observed in several CNS disorders, however their exact role remains unclear. Hence, several studies have shown a correlation of IL-16 with EAE (Skundric *et al*., 2005), MS (Skundric *et al*., 2015), SCI (Bank *et al*., 2015) and AD (Trombetta *et al*., 2018) in addition to our work on lymphocyte-derived IL-16-mediated neuroprotection (Shrestha *et al*., 2014). IL-16, previously reported as LCF, is classically known as a T-cell-derived cytokine that induces CD4-dependent migration and immune cell proliferation (Center *et al*., 1982, Parada *et al*., 1998, Liu *et al*., 1999). However, other immune cells have been reported to release IL-16, including monocytes (Elssner *et al*., 2004), eosinophils and mast cells (Laberge *et al*., 1999), epithelial cells (Cheng *et al*., 2001), DCs (Kaser *et al*., 1999), B cells (Sharma *et al*., 2000) and microglia (Guo *et al.*, 2004). Moreover, a neuronal variant, found only in the hippocampus and cerebellum, has been identified and characterized as a longer splice variant of the immune cell-derived IL-16 precursor protein, pro-IL-16, with two extra PDZ domains in the N-terminal region (Kurschner & Yuzaki., 1999). A study has shown that upon cleavage of the C-terminal region by caspase-3, mature IL-16 is released from NIL-16 transfected COS-7 cells (Kurschner & Yuzaki., 1999), similar to immune cell-derived IL-16. Furthermore, treatment of CGNs with mature IL-16 results in the CD4-dependent upregulation of the transcription factor cFos (Fenster *et al*., 2010), requiring tyrosine phosphorylation but not p38-MAPK (Kurschner & Yuzaki., 1999). It has also been shown to enhance neurite outgrowth, irrespective of CD4 expression (Fenster

et al., 2010). However, despite these findings, knowledge of if and how IL-16 modulates CNS function is still very limited. Therefore, I hypothesize that IL-16-containing LCM modulates neuronal and/or astrocytic activity. Hence, I aim to investigate whether LCM and recombinant IL-16 alters glutamate-, KCl- and/or ADP-induced intracellular Ca^{2+} changes in primary hippocampal cultures using live cell Ca^{2+} imaging techniques.

4.2 Results

4.2.1 Neurons and astrocytes are the predominant cell types present in mouse primary hippocampal cultures.

Immunostaining on 11-14 day old cultures confirmed neurons and astrocytes present in mouse primary hippocampal cultures cultured with AraC (5 µM; Figure 4.1 D), as standardly used for rat primary hippocampal cultures in our laboratory. However, astrocyte numbers in mouse cultures was reduced compared to rat cultures. Hence, varying AraC concentrations were added to mouse hippocampal cultures to determine ideal conditions. Increasing AraC concentrations (0 – 5 µM, Figure 4.1 A-D) resulted in decreased astrocyte numbers, but also reduced dendritic arbor development and formation of synaptic connections. Therefore, 1 µM AraC was determined visually as the ideal condition for mouse cultures (Figure 4.1 B), based on rat cultures. This concentration was then used in all subsequent cultures.

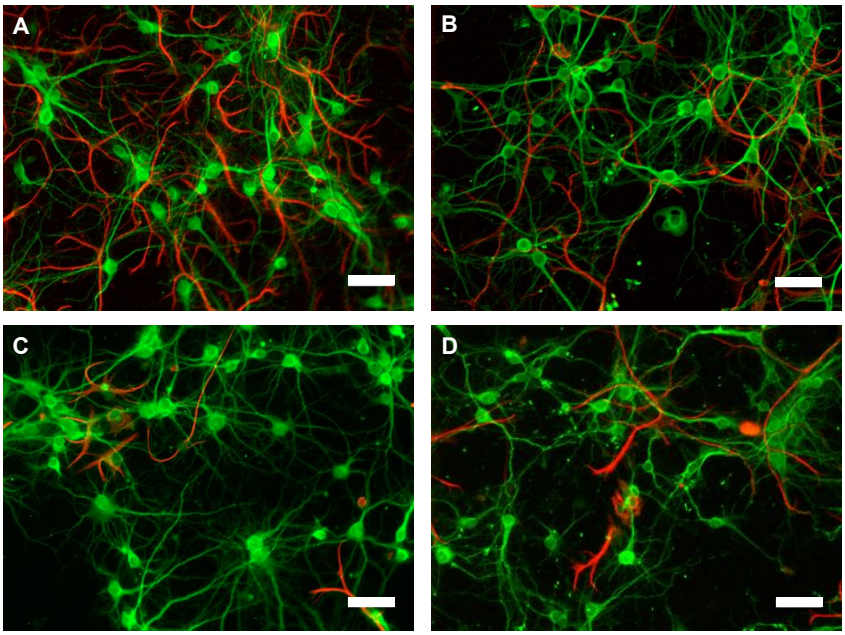

Figure 4.1. AraC (1 µM) best preserves astrocytic/neuronal ratio visually in mouse primary hippocampal cultures. Representaive images of MAP2-positive neurons (green) and GFAP-positive astrocytes (red) present in all cultures. (A) No AraC, (B) 1 µM, (C) 2 µM or (D) 5 µM AraC was added to C57BL/6J hippocampal cultures at 5 DIV. Data is a representative of three experiments. Scale bar = 50 µm.

4.2.2 Lymphocyte-conditioned medium impairs increases in neuronal intracellular calcium.

Given the presence of neurons and astrocytes in mouse primary hippocampal cultures, I next examined if neuronal and astrocytic activity was altered by the interaction with LCM and/or HLCM. Hence, live cell Ca^{2+} imaging revealed significantly reduced glutamate (10 μM)-induced increases in neuronal $[Ca^{2+}]_i$ ($n[Ca^{2+}]_i$) following exposure of hippocampal cultures to LCM (1 hour, 38.2 ± 20.5% increase, n=3, $p<0.05$ vs HCM control, Figure 4.2) but not HLCM (1 hour, 59.8 ± 1.7% increase, n=3, Figure 4.2) when compared to HCM control (125.2 ± 16.5% increase, n=, Figure 4.2). In contrast, KCl (20 mM)-induced increases in $n[Ca^{2+}]_i$ were not impaired following LCM exposure (1 hour, 26.7 ± 7.2% increase, n=3, $p<0.01$ vs HCM control, Figure 4.2) and HLCM exposure (1 hour, 44.7 ± 5.2% increase, n=3, Figure 4.2) in comparison to HCM control (53.4 ± 8.8% increase, n=3, Figure 4.2). Furthermore, ADP-application (20 μM) did not elicit any changes in $n[Ca^{2+}]_i$ in all experiments (n=3 HCM control treatment, n=3 LCM treatment and n=3 HLCM treatment, Figure 4.2A).

Having shown that LCM treatment impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases, I investigated the effects on pharmacologically-induced astrocytic $[Ca^{2+}]_i$ ($a[Ca^{2+}]_i$) increases using ADP (Begum et al., 2012) and glutamate (Xing et al., 2018). Astrocytes were deduced from their lack of response to KCl (Figure 4.3A). Live cell Ca^{2+} imaging revealed that LCM treatment (1 hour) was without effect on glutamate- and ADP-induced $a[Ca^{2+}]_i$ increases (glutamate: 35.9 ± 15.2% increase, ADP: 59.2 ± 17.8% increase, n=4, Figure 4.3) when compared to HCM control (glutamate: 47.0 ± 4.3% increase, ADP: 74.3 ± 12.8% increase, n=3, Figure 4.3). Analogous to LCM treatment, exposure to HLCM (1 hour) had no effect on ADP-induced increases in $a[Ca^{2+}]_i$ (46.3 ± 5.4% increase, n=3, Figure 4.3) or glutamate-induced $a[Ca^{2+}]_i$ increases (13.8 ± 5.5% increase, n=3, Figure 4.3) when compared to HCM control and LCM treatment.

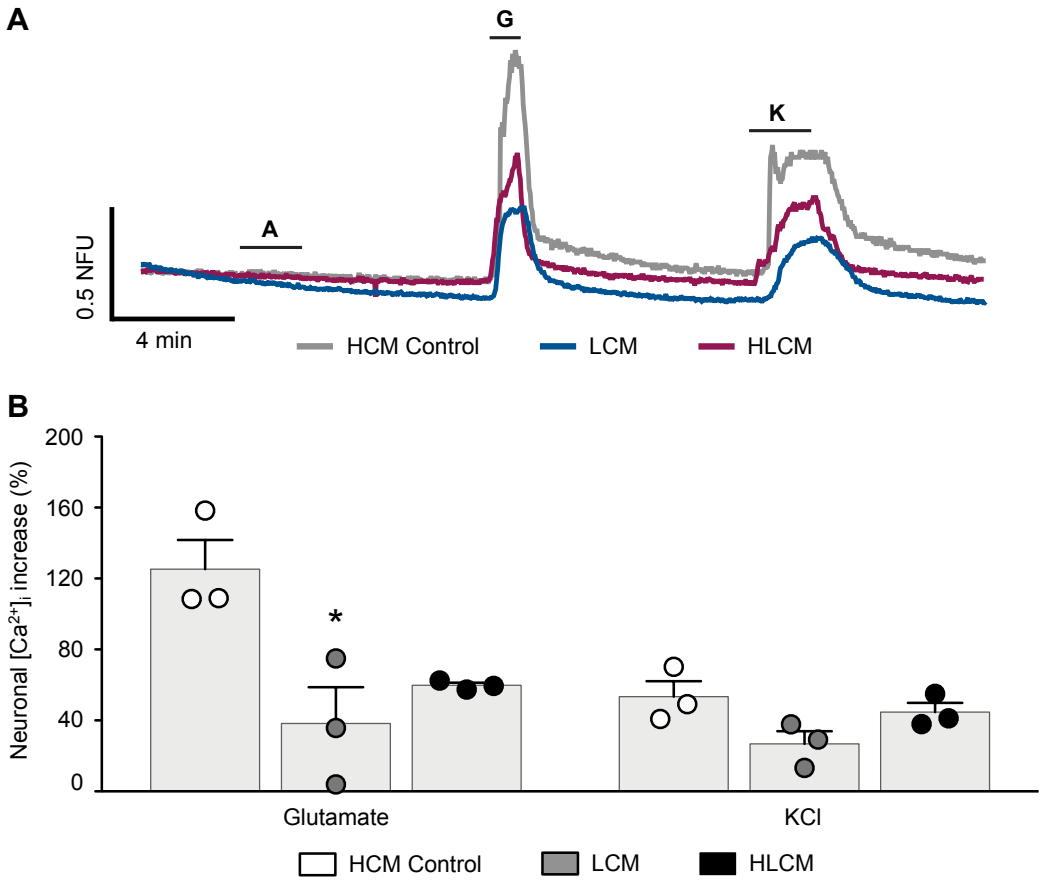

Figure 4.2. LCM but not HLCM treatment impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases. (A) Representative neuronal Ca^{2+} imaging traces of cultures exposed to HCM control, LCM or HLCM. (B) Bar chart with scatter plots illustrating LCM impairs neuronal glutamate-induced $[Ca^{2+}]_i$ responses. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. * = $p<0.05$ vs HCM control. All experiments were carried out in triplicate from three different C57BL/6J hippocampal cultures (n=3 HCM control treatment, n=3 LCM treatment and n=3 HLCM treatment). A = ADP, G = glutamate, K = KCl stimulation and NFU = normalised fluorescent unit.

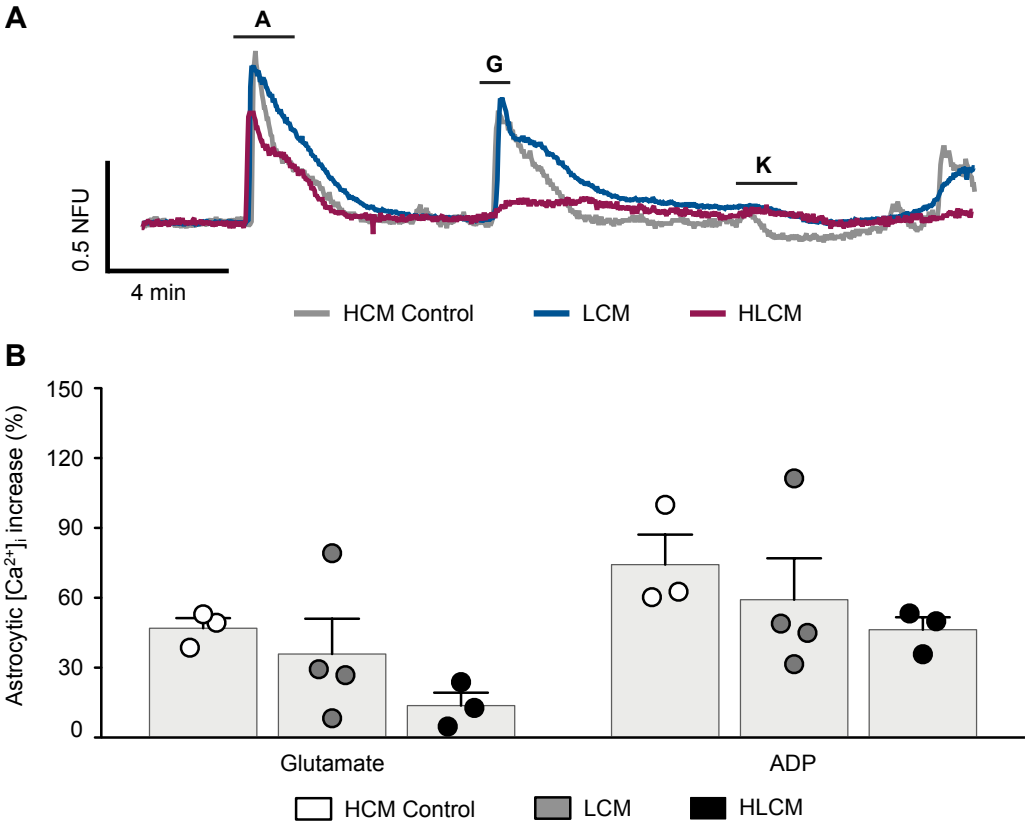

Figure 4.3. Both LCM and HLCM treatment do not impair pharmacological-induced astrocytic $[Ca^{2+}]_i$ increases. (A) Representative astrocytic Ca^{2+} imaging traces of cultures exposed to HCM control, LCM or HLCM. (B) Bar chart with scatter plots summarising no effects of LCM and HLCM on pharmacological-induced astrocytic $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. All experiments were carried out in triplicate from at least three different C57BL/6J hippocampal cultures (n=3 HCM control treatment, n=4 LCM treatment and n=3 HLCM treatment). A = ADP, G = glutamate, K = KCl stimulation and NFU = normalised fluorescent unit.

4.2.3 IL-16 is present in LCM and HLCM.

Having established that LCM treatment impairs glutamate-induced n$[Ca^{2+}]_i$ increases, I examined cytokine(s) responsible for the observed effect. In agreement with previous findings in our laboratory (Shrestha et al., 2014), both LCM (0 hours exposure: 360.0 ± 50.9 pg mL^{-1}, n=3, Figure 4.4) and HLCM (0 hours exposure: 90.0 ± 26.8 pg mL^{-1}, n=3, Figure 4.4) contained IL-16 following an 18-hour lymphocyte incubation and 24-hour hippocampal-lymphocyte co-incubation respectively, whereas no IL-16 was detected in HCM (0 hours exposure: 0 ± 0 pg mL^{-1}, n=3, Figure 4.4). Following LCM (1 hour exposure: 329.2 ± 38.0 pg mL^{-1}, n=3, Figure 4.4) and HLCM treatment (1 hour exposure: 86.1 ± 24.0 pg mL^{-1}, n=3, Figure 4.4), similar IL-16 levels were present but remained undetectable in HCM exposure (1 hour exposure: 0 ± 0 pg mL^{-1}, n=3, Figure 4.4). Hence, no significant difference was observed between treatment start and ending. Furthermore, I confirmed that the major immune-modulating cytokine IFN-γ was below detectable levels in all treatments (data not shown).

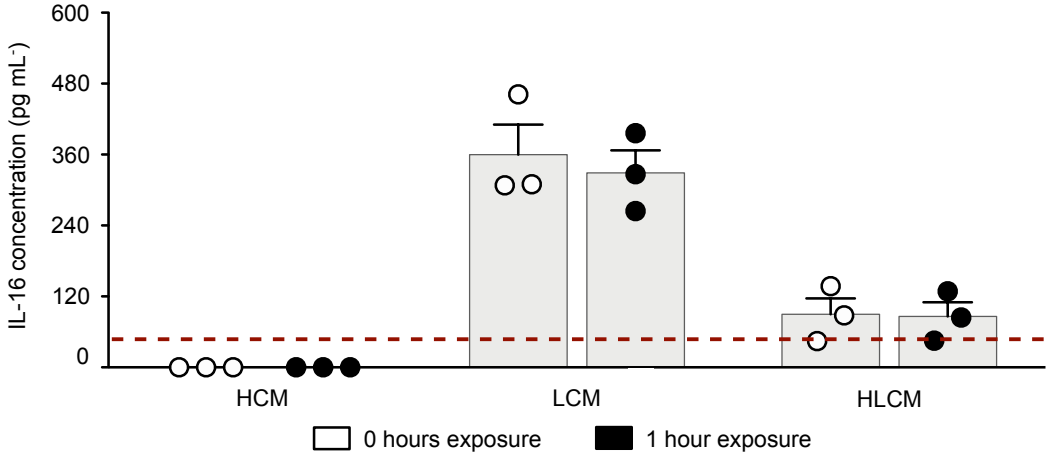

Figure 4.4. IL-16 is present in LCM and HLCM. (A & B) Bar chart with scatter plots revealing IL-16 is present in LCM and HLCM but not in HCM at the start of treatment (0 hours exposure) and is not degraded at the end of the 1-hour treatment. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t test. All experiments were carried out in triplicate from three different C57BL/6J lymphocyte preparations ($4x10^6$ cells mL^{-1}, n=3).

4.2.4 Short-term exposure to rIL-16 does not alter neuronal nor astrocytic intracellular calcium levels.

Having confirmed that IL-16 is present in LCM and HLCM, I used rIL-16 (300 pg mL^{-1}), similar to IL-16 levels in LCM, to confirm that IL-16 underlies the effects observed with LCM treatment. I examined first the effects of rIL-16 acute application on neuronal and astrocytic $[Ca^{2+}]_i$ levels, as previous studies have shown that IL-16 induces $[Ca^{2+}]_i$ increases in T cells (Cruikshank et al., 1991). Interestingly, live cell Ca^{2+} imaging revealed that acute rIL-16 application (300 pg mL^{-1}, 5 minutes) did not alter $n[Ca^{2+}]_i$ levels (-0.12 ± 0.4% increase, n=3, Figure 4.5) when compared to vehicle (0.7 ± 0.2% increase, n=3, Figure 4.5). In contrast, KCl induced increases in $n[Ca^{2+}]_i$ levels (66.0 ± 8.3% increase, n=3 neurons, $p<0.001$ vs vehicle, Figure 4.5) when compared to vehicle.

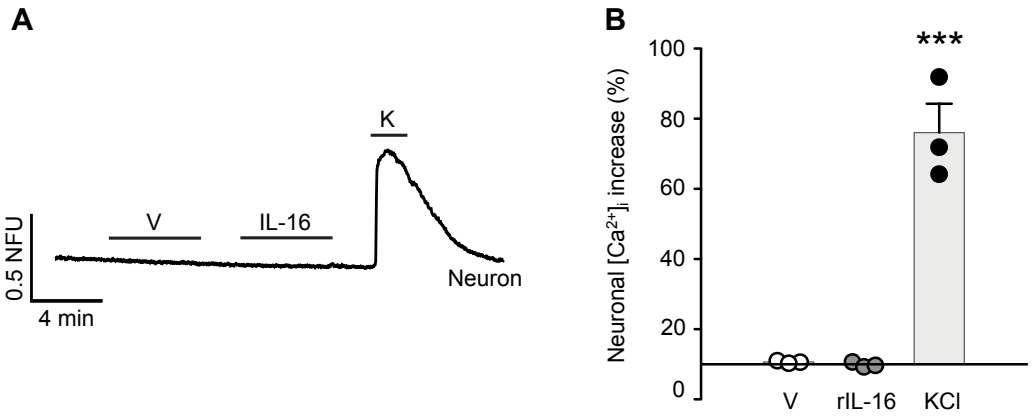

Figure 4.5. Acute rIL-16 application does not alter neuronal $[Ca^{2+}]_i$ levels. (A) Representative neuronal Ca^{2+} imaging trace illustrating acute exposure to vehicle, rIL-16 and KCl. (B) Bar chart with scatter plots summarising no effect of acute exposure to rIL-16 on neuronal $[Ca^{2+}]_i$ levels. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. *** = $p<0.001$ vs vehicle. All experiments were carried out in triplicate from three different C57BL/6J hippocampal cultures (n=3). V = vehicle, K = KCl stimulation and NFU = normalised fluorescent unit.

Whilst the overall effect of rIL-16 on a$[Ca^{2+}]_i$ levels was absent (15.7 ± 17.8% increase, n=3 astrocytes, Figure 4.6B) when compared to vehicle (1.1 ± 2.2% increase, n=3, Figure 4.6B), in 4 out of 18 astrocytes, rIL-16 induced $[Ca^{2+}]_i$ increases (Figure 4.6A).

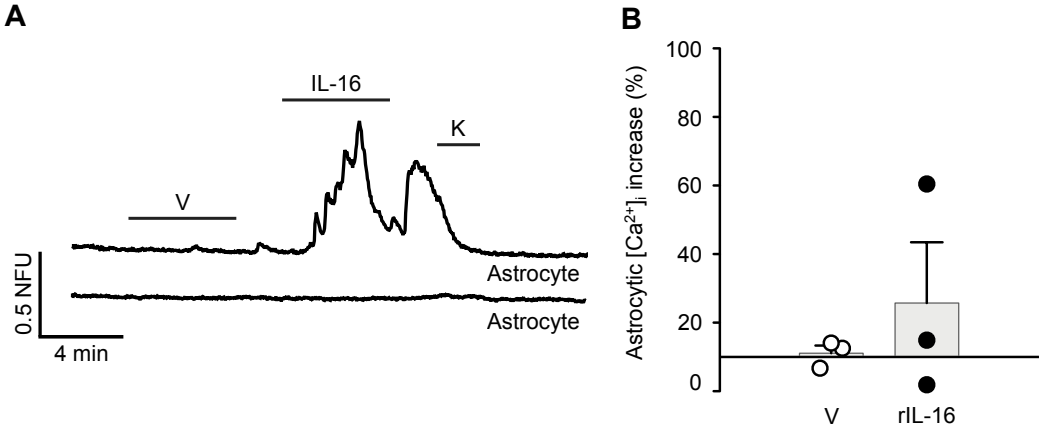

Figure 4.6. Acute rIL-16 application does not alter astrocytic $[Ca^{2+}]_i$ levels. (A) Representative astrocytic Ca^{2+} imaging traces illustrating acute exposure to vehicle, rIL-16 and KCl. (B) Bar chart with scatter plots summarising no effect of acute exposure to rIL-16 on astrocytic $[Ca^{2+}]_i$ levels. Data are presented as mean ± S.E.M. and analysed using a unpaired Student's t test. All experiments were carried out in triplicate from three different C57BL/6J hippocampal cultures (n=3). V = vehicle, K = KCl stimulation and NFU = normalised fluorescent unit.

4.2.5 rIL-16 impairs glutamate-induced neuronal intracellular calcium responses.

Having shown that acute rIL-16 application has no effect on neuronal nor astrocytic $[Ca^{2+}]_i$ levels, I investigated the long-term exposure (1 hour) effects of IL-16. Comparable to LCM treatment, live cell Ca^{2+} imaging revealed that exposure to rIL-16 (300 pg mL^{-1}, 1 hour) impaired glutamate-induced increases in n$[Ca^{2+}]_i$ (52.3 ± 13.4% increase, n=4, Figure 4.7), but was not significant when compared to vehicle treatment (63.3 ± 13.6% increase, n=4, Figure 4.7). This effect was partially abolished when rIL-16 was denatured (dIL-16) prior to treatment (56.3 ± 2.8% increase, n=4, Figure 4.7). Analogous to LCM and HLCM treatment, no effect

was observed on KCl-induced n[Ca^{2+}]$_i$ increases following rIL-16 exposure (300 pg mL^{-1}, 1 hour, 56.6 ± 6.3% increase, n=3, Figure 4.7) when compared to vehicle treatment (46.2 ± 1.6% increase, n=3, Figure 4.7). Exposure to dIL-16 had no effect either on KCl-induced n[Ca^{2+}]$_i$ increases (60.4 ± 8.9% increase, n=3, Figure 4.7) when compared to vehicle and rIL-16 treatment. Furthermore, ADP-application did not elicit any changes in n[Ca^{2+}]$_i$ in all experiments (n=4 vehicle treatment, n=4 rIL-16 treatment and n=4 dIL-16 treatment, Figure 4.7A).

Having established that rIL-16 exposure shows a trend towards impaired neuronal glutamate-induced [Ca^{2+}]$_i$ increases, I examined its effect on astrocytic glutamate- and ADP-induced [Ca^{2+}]$_i$ increases. Again, astrocytes were deduced from their lack of response to KCl (Figure 4.8A). Live cell Ca^{2+} imaging showed that rIL-16 exposure (300 pg mL^{-1},1 hour) was without effect on both glutamate- and ADP-induced a[Ca^{2+}]$_i$ increases (glutamate: 28.8 ± 6.5% increase, ADP: 33.3 ± 7.4% increase, n=4, Figure 4.8) when compared to vehicle treatment (glutamate: 26.9 ± 6.4% increase, ADP: 39.1 ± 2.7% increase, n=4, Figure 4.8). Furthermore, no effect was observed when exposed to dIL-16 (glutamate: 26.9 ± 8.4% increase, ADP: 50.2 ± 5.8% increase, n=4, Figure 4.8).

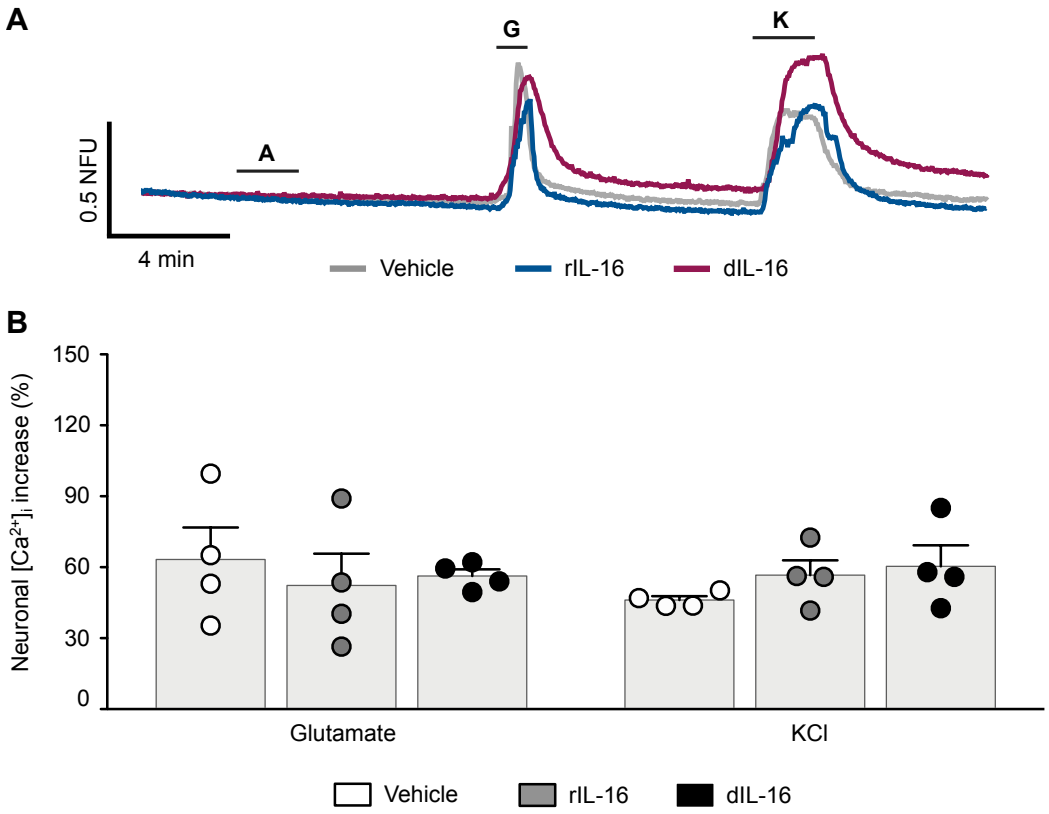

Figure 4.7. rIL-16 shows a trend towards impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases following a 1-hour exposure. (A) Representative neuronal Ca^{2+} imaging traces of cultures exposed to vehicle, rIL-16 or dIL-16. (B) Bar chart with scatter plots summarising that rIL-16 impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases and dIL-16 enhances KCl-induced neuronal $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. All experiments were carried out in triplicate from four different C57BL/6J hippocampal cultures (n=4 vehicle treatment, n=4 rIL-16 treatment and n=4 dIL-16 treatment). A = ADP, G = glutamate, K = KCl stimulation and NFU = normalised fluorescent unit.

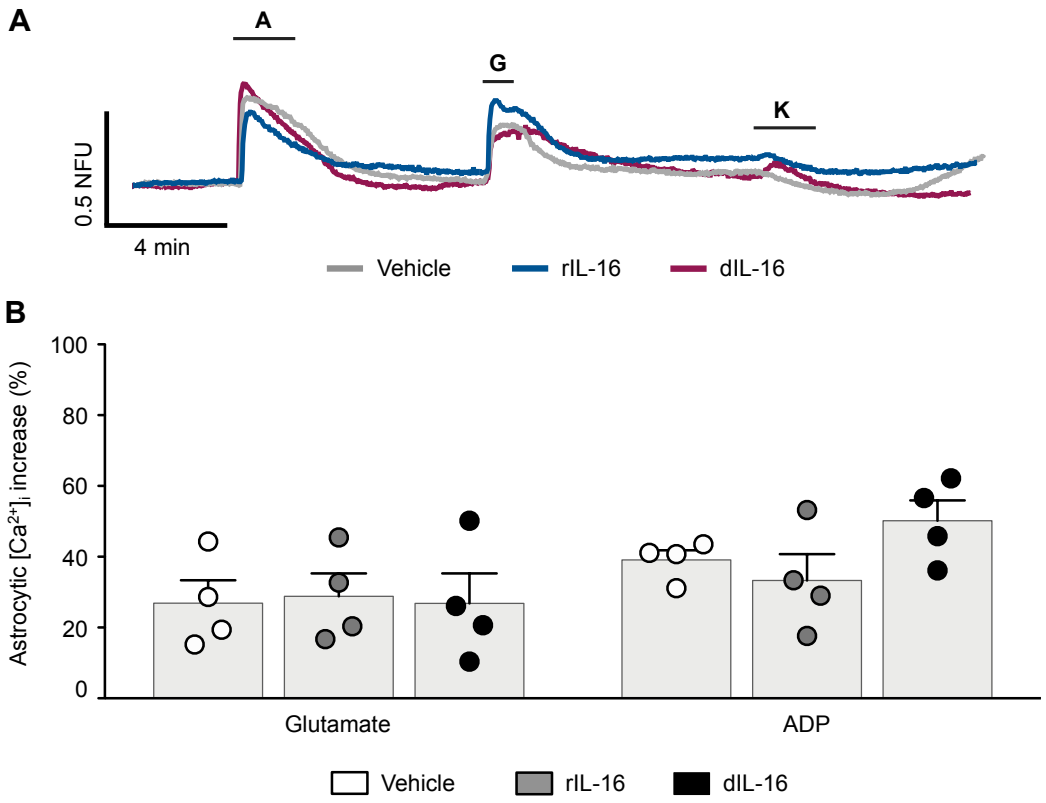

Figure 4.8. Glutamate- and ADP-induced astrocytic $[Ca^{2+}]_i$ increases are unchanged following a 1-hour rIL-16 exposure. (A) Representative astrocytic Ca^{2+} imaging traces of cultures exposed to vehicle, rIL-16 or dIL-16. (B) Bar chart with scatter plots summarising no effect of rIL-16 on glutamate- or ADP-induced astrocytic $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni post-hoc comparison. All experiments were carried out in triplicate from four different C57BL/6J hippocampal cultures (n=4 vehicle treatment, n=4 rIL-16 treatment and n=4 dIL-16 treatment). A = ADP, G = glutamate, K = KCl stimulation and NFU = normalised fluorescent unit.

4.2.6 Mouse hippocampal neurons and astrocytes do not express CD4.

Given that it has been reported previously that the actions of IL-16 in CGNs are both CD4-dependent and -independent (Fenster et al., 2010), I examined CD4 expression in the C57BL/6J primary hippocampal cultures. Immunostaining revealed that no CD4 expression was evident in 11-14 day old cultured hippocampal neurons (Figure 4.9A & B) nor astrocytes

(Figure 4.9C & D), whereas positive CD4 staining was observed in control lymphocyte preparations (Figure 4.9E-G).

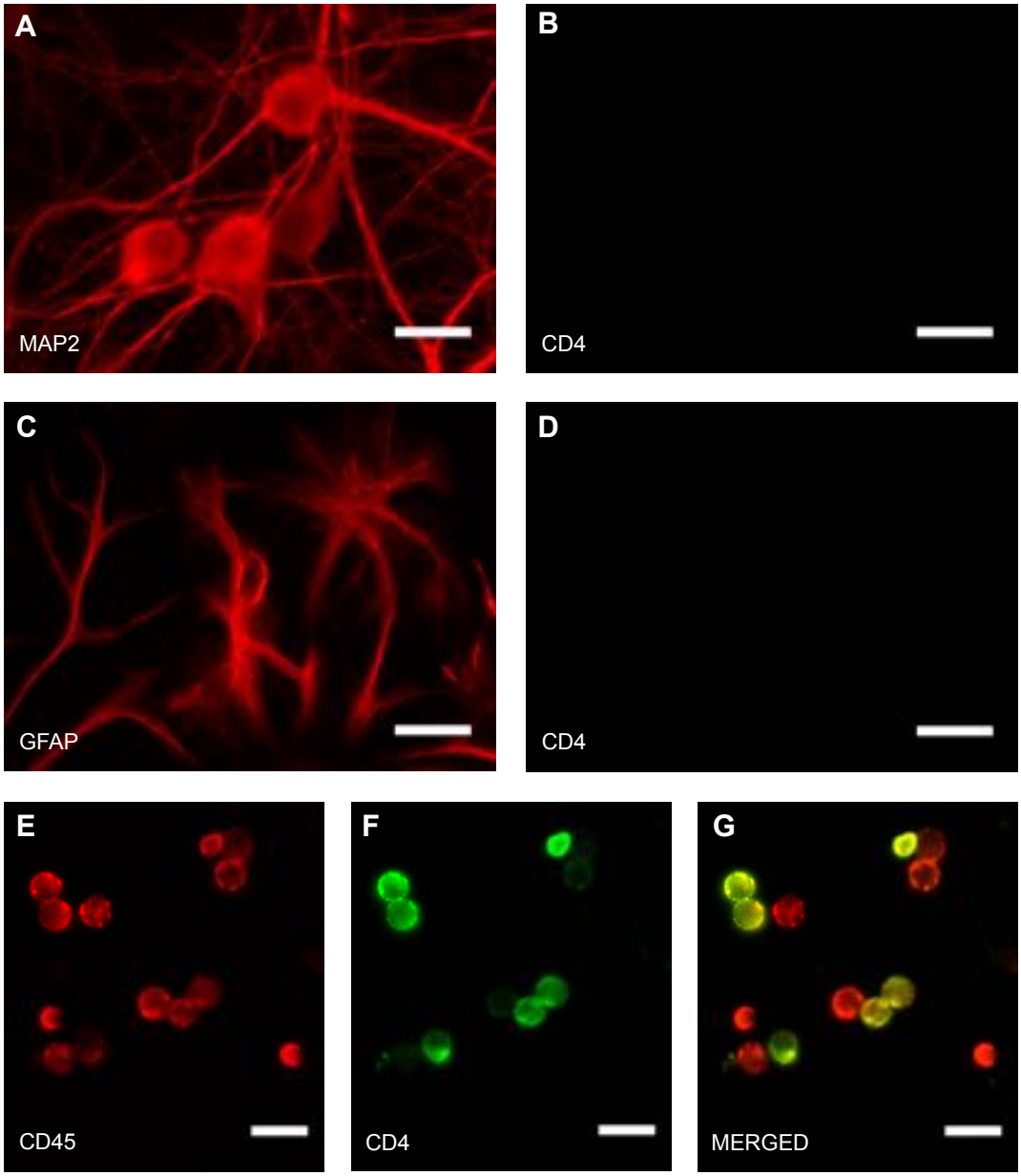

Figure 4.9. CD4 receptor is not expressed in mouse primary hippocampal cultures. (A-D) Representative images showing CD4 (green) is not present on MAP2-positive neurons (red) nor GFAP-positive astrocytes (red) in C57BL/6J hippocampal cultures. (E-G) Representative images confirming CD4 (green) expression on CD45-positive lymphocytes (red). Data is a representative of four experiments. Scale bar = 20 μm (A-D) and 10 μm (E-G).

4.3 Discussion

In order to better understand the role of inflammatory mediators on neuronal and astrocytic function, I treated hippocampal cells with LCM or HLCM. My data reveals that LCM impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases, which may be due to IL-16-induced modulation of neuronal glutamate receptors. Furthermore, this effect of LCM on neuronal function was CD4-independent, as CD4 was absent in hippocampal cells under my experimental conditions.

4.3.1 Neuronal and astrocyte preservation in mouse hippocampal cultures.

Immunocytochemistry confirmed that both neurons (MAP2) and astrocytes (GFAP) are present in my mouse hippocampal cultures. Astrocytes are needed in sufficient amounts to secrete essential factors that facilitate neuronal and synaptic development (Allen *et al.*, 2012; van Deijk *et al.*, 2017), including synapse remodelling (Chung *et al.*, 2013). Furthermore, it is proposed that astrocytes can regulate neuronal membrane excitability via Ca^{2+} elevations leading to gliotransmitter release, but this is an area of intense debate (Hassinger *et al.*, 1995; Fiacco *et al.*, 2007; Pirttimaki *et al.*, 2017; Fiacco and McCarthy, 2018). However, in hippocampal cell culture experiments, when hippocampal tissue is dissociated, astrocytes become activated and start to proliferate rapidly, leading to an astrocyte overgrowth (Rigato, 2005). To prevent that astrocytes exponentially compete with developing neurons, anti-mitotic agents are added, reducing the population of proliferating cells, capable of DNA synthesis (Delivopoulos and Murray, 2011). In my project, Ara-C was tested at different concentrations to reduce extensive astrocyte proliferation and preserve neuronal conformity at the same time. Our laboratory has set the lowest effective dose for rat primary hippocampal cultures to 5 μM, added at 5 DIV. However, adding this Ara-C concentration to mouse hippocampal cultures at 5 DIV, astrocyte numbers are significantly reduced, and this concentration is toxic to neurons, visualised by a reduction in dendritic arbor development and a reduced formation of synaptic connections. These neurotoxic effects were similarly observed in DRG neurons (Wallace and Johnson, 1989; Zhuo, *et al*., 2018) as well as several other neuronal cell types, including

sympathetic neurons (Tomkins, *et al*., 1994), cerebellar neurons (Dessi *et al.*, 1995; Koros and Kitraki, 2014) and spinal cord motoneurons (Sanz-Rodriguez, Boix and Comella, 1997; Audet *et al.*, 2012). As neurons are post-mitotic cells, neurotoxic effects are not induced by premature termination of DNA elongation, introducing DNA strand breakage and cell death as seen in proliferating cells (Garcia-Diaz *et al.*, 2010). Several mechanisms of action have been introduced, with interference of nerve growth factors being one of the first (Martin, *et al*., 1990). Nowadays, it is reported that AraC produces ROS, which then induce oxidative DNA damage and neuronal apoptosis (Geller and Cheng, 2001; Zhuo, *et al*., 2018). To prevent neurotoxicity and at the same time excessive glial proliferation, Ara-C concentration was lowered to 1 μM, which preserved neuronal and glial cell conformity, as MAP2 staining showed that hippocampal neurons were appropriately polarized, developed extensive dendritic arbors and formed numerous synaptic connections at this concentration.

4.3.2 LCM and HLCM impair increases in neuronal intracellular calcium.

As previously shown by our laboratory, lymphocytes placed beneath the OSC culture membrane as well as LCM prevented kainate-induced neurotoxicity (Shrestha *et al*., 2014). Similar to the work in our laboratory, another study showed that MBP- and OVA-specific T cells were able to protect neurons from secondary degeneration in a contact-independent manner by placing the T cells beneath the OSC culture membrane (Wolf *et al*., 2002). Both studies suggest that this neuroprotection is mediated by the action of soluble inflammatory mediators on either neurons or astrocytes, however exact mechanisms are still unknown. Therefore, to better understand soluble mediator action(s) on neurons and/or astrocytes, I investigated whether LCM modulates pharmacologically-induced intracellular calcium increases in neurons and astrocytes from hippocampal cell cultures. Hence, to the best of my knowledge, I have shown for the first time that LCM treatment impairs glutamate-induced increases in n$[Ca^{2+}]_i$. It is unlikely that the impaired neuronal activity is due to glutamate-induced cell death, as cells fully recover following glutamate application, seen by $[Ca^{2+}]_i$ levels

returning back to baseline. Interestingly, exposure to LCM showed a neuron-specific effect as it only interfered with glutamate-induced neuronal intracellular calcium signalling. This treatment could suggest a protective role against glutamate-induced neuronal excitotoxicity by dampening the influx of calcium in response to glutamate, as an overload of calcium activates a number of enzymes that damages cellular structures (Lee *et al.*, 2018; Müller *et al.*, 2018). My data also suggest that LCM and HLCM act differently on neurons and astrocytes and therefore the neuroprotection found, by the previous work in our laboratory, with both treatments could have been induced via different mechanisms.

To better understand these distinctive effects, IL-16 levels were determined in LCM or HLCM, as it was previously shown that the neuroprotective effects were driven by the cytokine IL-16 (Shrestha *et al*., 2014). In agreement, both LCM and HLCM contained IL-16 levels, but were absent in HCM. Other cytokine levels, including IFN-γ were below detectable levels in all three conditions, which agrees with other studies showing that lymphocytes mainly secrete cytokines upon activation and hippocampal cultures do not secrete classical cytokines (Ritchie *et al*., 2018). IL-16 levels, however were significantly lower in HLCM. This could be due to hippocampal cells blocking IL-16 release from lymphocytes or hippocampal cells taking up secreted IL-16. Which one of these explanations account for the difference in IL-16 observed is not known, as to the best of my knowledge no studies have investigated the effect of hippocampal cells on IL-16 release from lymphocytes. It was, however confirmed that within 1 hour *in vitro* hippocampal cells do not take up IL-16, as by the end of LCM and HLCM treatment of hippocampal cells, IL-16 levels were not significantly decreased. Further experiments would need to be conducted to confirm that hippocampal cells do not take up IL-16 over longer periods of time (within a 24-hour time frame).

4.3.3 IL-16 might underlie the effects observed with LCM treatment.

As IL-16 levels in LCM were quantified at approximately 300 pg mL^{-1}, I used rIL-16 at this concentration to examine whether IL-16 underlies the effects seen with LCM treatment. As previous studies have shown that IL-16 induces increases in $[Ca^{2+}]_i$ in T cells via InsP3 generation in a CD4-dependent manner (Cruikshank *et al*., 1991), I determined whether acute application of rIL-16 (5 minutes) modulates neuronal and astrocytic $[Ca^{2+}]_i$ levels. In contrast to T cells, acute rIL-16 application did not induce an increase in either neuronal nor astrocytic $[Ca^{2+}]_i$ levels (Hridi et al., 2019). To fully confirm this, further experiments would be required that either use the IL-16 concentration that is used in T cells (1µg mL^{-1}, Cruikshank *et al*., 1991) to induce a calcium flux in hippocampal cells or investigate the concentration used in my experimental conditions (300 pg mL^{-1}) on T cells and see if this is sufficient to induce $[Ca^{2+}]_i$ changes.

To further assess the effects of IL-16 on hippocampal cells, I exposed hippocampal cultures to rIL-16 for a longer period (1 hour), similar to LCM and HLCM treatment. Exposure of hippocampal cultures to rIL-16 for 1 hour resulted in reduced glutamate-induced $n[Ca^{2+}]_i$ increases (Hridi et al., 2019), however this effect only showed a trend and was thus not significant, as observed with LCM. It has to be mentioned though that the glutamate responses following HCM control were much lower in the rIL-16 experiment (60% neuronal intracellular calcium increase) than in the LCM/HLCM experiment (120% neuronal intracellular calcium increase). This was however attributed to cultures being not as healthy as previous LCM/HLCM experiments, as also explained in section 5.3.2. Thus, this might explain why the reduction in glutamate-induced $n[Ca^{2+}]_i$ increases was not significant compared to its control. Furthermore, no effects were observed on KCl-induced $n[Ca^{2+}]_i$ increases and glutamate- and ADP-induced $a[Ca^{2+}]_i$ increases (Hridi et al., 2019). This data suggests that IL-16 might underlie the effects observed with exposure to LCM. To the best of my knowledge, this is the first study looking at IL-16 effects on hippocampal Ca^{2+} modulation. However, other cytokines

have been shown to modulate intracellular Ca^{2+} levels. Indeed, TNF-α reduces glutamate-induced $[Ca^{2+}]_i$ increases in astrocytes, while other cytokines, including IL-1ß, IL-2 and IL-6 were without effect (Köller *et al.*, 2001). In addition, IL-6 decreases neuronal NMDAR-mediated Ca^{2+} overload, preventing neuronal death by inhibiting NR2B and NR2C subtype receptor activities (Ma *et al.*, 2015). Furthermore, IFN-γ mediates neuroprotection against glutamate-excitotoxicity by enhancing the recovery of neuronal glutamate-induced $[Ca^{2+}]_i$ increases (Lee *et al.*, 2006). Thus, there is clear evidence that cytokines modulate glutamate receptors, similar to the data shown in this chapter regarding IL-16 exposure. However, it is not known whether the underlying mechanisms are the same. The use of transcriptomics could provide more insight into this, however this was beyond the scope of my PhD.

Further research in our laboratory has confirmed this modulation of glutamate receptors via IL-16. As such, IL-16 exposure to hippocampal cultures, using the same conditions that impaired glutamate-induced $n[Ca^{2+}]_i$ increases following LCM treatment, resulted in decreased spontaneous EPSC (sEPSC) frequency and amplitude (Hridi et al., 2019). As changes in excitatory synaptic transmission are accepted to be primarily mediated by changes in AMPA receptor numbers or kinetics (Huganir and Nicoll, 2013; Gu *et al.*, 2016), involvement of postsynaptic GluA1 AMPA receptor (AMPAR) was further confirmed by showing that GluA1 S831 phosphorylation was impaired upon IL-16 exposure (1 hour, Hridi et al., 2019). Given that phosphorylation of GluA1 at the S831 site is known to be induced by calcium/CaMKII and PKC (Mammen *et al.*, 1997; Kristensen *et al.*, 2011; Ren *et al.*, 2013), it could be implied that PKC translocation is downregulated in hippocampal neurons upon IL-16 exposure. This is in contrast to a rapid translocation of PKC found in IL-16-induced migratory responses of $CD4^+$ normal blood T cells, SUPT1 cells and THP1 cells (Parada *et al.*, 1996). Hence, it is unknown if and how this PKC pathway relates to the effects observed by IL-16 exposure to hippocampal cells and therefore further experiments need to be conducted examining PKC translocation in hippocampal neurons after IL-16 exposure. To the best of my

knowledge, this is the first time that IL-16 has been shown to interact with AMPARs. In contrast, NIL-16 has been known to co-immunoprecipitate with NMDARs, in particular all NR2A-D subtypes (Kurschner and Yuzaki, 1999). However, this interaction is dependent on the presence of PDZ domains in the N-terminal part of NIL-16, which is not present in immune-cell derived IL-16. Furthermore, in addition to IL-16, IFN-γ has been shown to interfere with GluA1 (Mizuno *et al.*, 2008). However, this was via S845 phosphorylation, and therefore it is possible that IFN-γ function is different from IL-16 and different pathways are involved.

4.3.4 LCM-induced impaired neuronal intracellular calcium increases are CD4-independent.

Given that acute application of rIL-16 does not induce increases in neuronal nor astrocytic $[Ca^{2+}]_i$, which is known to be a CD4-dependent mechanism in T cells (Cruikshank *et al*., 1991), and that the actions of IL-16 in CGNs are both CD4-dependent and -independent (Fenster *et al*., 2010), I examined whether CD4 is expressed by hippocampal cells. I show that CD4 was not present on neurons or astrocytes under my experimental conditions. This is in contrast with a few studies showing CD4 expression on CNS parenchymal cells, including neurons and astrocytes in human brains (Funke *et al.*, 1987), microglia in normal rat brains (Perry and Gordon, 1987) and neurons in adult mouse brains (Omri *et al.*, 1994). However, studies focussing on CNS cells involved in HIV infections have shown that microglia and macrophages are the only cell types present in the CNS that express CD4, the receptor required for cells to become infected with viral glycoproteins, inducing HIV (González-Scarano and Martín-García, 2005). Furthermore, CD4 microglial expression has been noticeably associated with diseases involving an immune component including EAE (Almolda *et al.*, 2009). Considering this, it is still under debate whether CNS cell types express CD4. My stainings, however support absent effects on basal $[Ca^{2+}]_i$ levels as IL-16 induces increases in $[Ca^{2+}]_i$ in T cells via a CD4-dependent mechanism and suggests that the impaired effects on glutamate-

induced increases in n$[Ca^{2+}]_i$ are CD4-independent. It has indeed been shown that NIL-16 modulates neuronal function via CD4-dependent and -independent mechanisms (Kurschner and Yuzaki, 1999, Fenster *et al*., 2010). IL-16-induced increases in c-fos expression were CD4-dependent, as CD4-deficient CGNs did not upregulate c-fos expression. However, IL-16 did enhance neurite outgrowth in CD4-deficient CGNs similar to CD4-positive CGNs (Fenster *et al*., 2010). Furthermore, T cells from CD4-knockout mice have been shown to perform chemotaxis and produce inflammatory cytokines following contact with IL-16 (Mathy *et al.*, 2000), suggesting that CD4 is not the only receptor that interacts with IL-16. Thus, work presented in this chapter and other work performed in our laboratory, further supports this perspective. However, to fully confirm that the impaired glutamate-induced increases in n$[Ca^{2+}]_i$ are CD4-independent, hippocampal cultures from CD4-knockout mice would be required to test the effects of LCM and IL-16 on hippocampal calcium signalling. Furthermore, it has been shown that IL-16 induces migration in human lung epithelial cells (A549) via the CD9 receptor (Blake *et al.*, 2018). Therefore, it would be interesting to test for this receptor in mouse hippocampal cultures used for these experiments. Interestingly, our laboratory has shown via immunofluorescent stainings and western blotting that CD9 receptors were not expressed on hippocampal neurons nor astrocytes (Hridi et al., 2019). Hence, more experiments would need to be performed to unravel whether the GluA1 receptor is directly or indirectly targeted in LCM- and IL-16-mediated reduction in GluA1 functioning.

In conclusion, data in this chapter suggest that cytokines, possibly IL-16, might underlie impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases following LCM. This extends our knowledge of how cytokines and IL-16 in particular modulates CNS function. Together with data from our laboratory it is suggested that IL-16 modulates GluA1 receptors via CD4- and CD9-independent mechanisms.

Chapter 5

Determining contact-dependent effects of lymphocytes on neuronal synaptic communication and astrocyte function in hippocampal cultures using microfluidic devices.

5.1 Introduction

While cytokines can affect neuronal activity (see chapter 4) and thus maybe disease progression and outcome, increased lymphocyte numbers have also been found in many CNS diseases, including MS, stroke and AD (Denic, Wootla and Rodriguez, 2013; Ruhnau *et al.*, 2016; Merlini *et al.*, 2018). Lymphocytes are primarily known to contribute to disease progression by modulating neuronal function via contact-dependent mechanisms (Liblau *et al.*, 2013). Hence, it has been shown that $CD4^+$ Th cells mediate dopaminergic-induced neuronal toxicity in a PD and AD mouse model via FasL/Fas contact-dependent interactions (Brochard *et al.*, 2009; Zhang *et al.*, 2013). Furthermore, activated T cells have been shown to induce cytotoxicity in human neuronal cultures in a contact-dependent manner, involving FasL, LFA-1 and CD40 but do this independently of antigen and MHC I (Giuliani *et al.*, 2003). It has also been shown that PLP- and ovalbumin-specific $CD4^+$ T cells directly induce lethal neuronal Ca^{2+} increases in murine acute brain slices (Nitsch *et al*., 2004). This contact-dependent interaction was MHC-independent and was prevented by blocking perforin and glutamate receptors. In addition, ovalbumin-specific $CD8^+$ T cells induce apoptosis in MHC-I expressing, ovalbumin presenting hippocampal neurons via perforin-mediated delivery of granzymes (Meuth *et al*, 2009). Whilst these studies have advanced our understanding of how T cells may contribute to cell death, these studies reveal many different contact-dependent mechanisms involved and therefore their controversial role requires more research.

To monitor functional connectivity of primary hippocampal co-culture networks and thereby synaptic communication, our laboratory has developed an *in vitro* system that integrates microfluidics with Ca^{2+} imaging techniques (Robertson *et al*., 2014; Samson *et al*., 2016, MacKerron *et al*., 2017). While many studies use microfluidic platforms to address their neurological questions (Neto *et al.*, 2016; Osaki *et al.*, 2017; Fantuzzo *et al.*, 2018; Wnorowski, Yang and Wu, 2018), many questions remain regarding interactions between multiple neuronal networks, also referred to as synaptic communication. Thus, platforms

designed in our laboratory allow for the investigation of how the activity of one hippocampal cultured network is modulated by changes in activity in a second, environmentally isolated, network via functional synaptic connections. Hence, I hypothesize that lymphocytes modulate neuronal and/or astrocytic activity as well as synaptic communication in a contact-dependent manner. In this chapter, I therefore aim to examine whether lymphocytes alter glutamate-, KCl- and/or ADP-induced intracellular Ca^{2+} changes in primary hippocampal cultures by integrating microfluidics with live cell Ca^{2+} imaging techniques.

In addition to addressing consequences of lymphocytes on synaptic communication, this platform is also a useful tool to investigate lymphocyte migratory behaviour within the CNS. Whilst it is known how immune cells infiltrate the CNS via its three distinct routes, including the endothelial BBB, blood-CSF barrier and meningeal barrier (Wilson, *et al*., 2010; Engelhardt and Ransohoff, 2012), many questions remain regarding what happens to immune cells once infiltrated into CNS parenchyma. To study cell migration, Boyden-like transwell assays are commonly used, however their capacity is limited by poor compound gradient control and an inability to visualise cell migration (Keenan and Folch, 2008), and therefore importantly this technique cannot distinguish between enhanced cell motility and migratory persistence. Furthermore, in this system, cells migrate only towards certain stimuli, including chemoattractants or cytokines (Kobayashi *et al.*, 2017). Thus, this method does not allow for simulation of tissue-level physiology and therefore cellular alterations, including neuronal cell death or activity, which might induce cell migration, cannot be studied. Hence, I hypothesize that neuronal activity and/or neuronal cell death changes lymphocyte migration behaviour. I therefore also aim to examine in this chaptor the possibility of using microfluidic devices to study lymphocyte migratory behaviour following glutamate-, KCl- and/or ADP-induced intracellular Ca^{2+} increases or glutamate-induced cell death.

5.2 Results

5.2.1 IL-16 and IFN-γ are not present in lymphocyte preparations.

To confirm that the effects of lymphocytes on pharmacologically-induced $[Ca^{2+}]_i$ increases were via contact-dependent mechanism, I determined IL-16 and IFN-γ cytokine levels during exposure of hippocampal cultures to lymphocytes. In agreement with the results in chapter 4, both IL-16 (0 hours exposure: 0 ± 0 pg mL^{-1}, 1 hour exposure: 0 ± 0 pg mL^{-1}, n=3, Figure 5.1A) and IFN-γ levels (0 hours exposure: 0 ± 0 pg mL^{-1}, 1 hour exposure: 0 ± 0 pg mL^{-1}, n=3, Figure 5.1B) remained undetectable in HCM control during exposure of hippocampal cultures. When hippocampal cultures were exposed to lymphocytes (1 hour), both IL-16 (0 hours exposure: 8.4 ± 8.4 pg mL^{-1}, 1 hour exposure: 1.5 ± 1.5 pg mL^{-1}, n=3, Figure 5.1A) and IFN-γ levels (0 hour exposure: 0.0 ± 0.0 pg mL^{-1}, 1 hour exposure: 0.0 ± 0.0 pg mL^{-1}, n=3, Figure 5.1B) were not detected. Hence, IL-16 and IFN-γ cytokine levels were not significantly increased during lymphocyte exposure.

5.2.2 Lymphocytes have no effect on neuronal or astrocytic intracellular calcium responses.

Having established previously that LCM impairs glutamate-induced $n[Ca^{2+}]_i$ increases (see chapter 4), I investigated contact-dependent effects of lymphocytes on neuronal and astrocytic activity. Hence, following exposure of hippocampal cultures on coverslips to lymphocytes directly (1 hour), live cell Ca^{2+} imaging revealed that glutamate (10 μM)-induced increases in $n[Ca^{2+}]_i$ (46.6 ± 11.8% increase, n=2, Figure 5.2) were reduced when compared to HCM control (90.5 ± 41.1% increase, n=3, Figure 5.2), however this effect was not significant. Furthermore, KCl (25 mM)-induced increases in $n[Ca^{2+}]_i$ following lymphocyte exposure (1 hour, 37.8 ± 6.1% increase, n=2, Figure 5.2) were not significantly different from HCM control (48.9 ± 4.5% increase, n=3, Figure 5.2). In addition, ADP (20 μM) did not elicit any changes in $n[Ca^{2+}]_i$ in all experiments (n=3 HCM control treatment and n=2 lymphocyte treatment,

Figure 5.2A). In contrast, ADP and glutamate application resulted in increased a$[Ca^{2+}]_i$ levels following HCM control exposure (ADP: 68.9 ± 7.2% increase, glutamate: 48.4 ± 14.4% increase, n=3, Figure 5.3). However, compared to HCM control, ADP- and glutamate-induced increases in a$[Ca^{2+}]_i$ were not significantly different following lymphocyte exposure (ADP: 67.8 ± 13.3% increase, glutamate: 37.6 ± 4.3% increase, n=3, Figure 5.3).

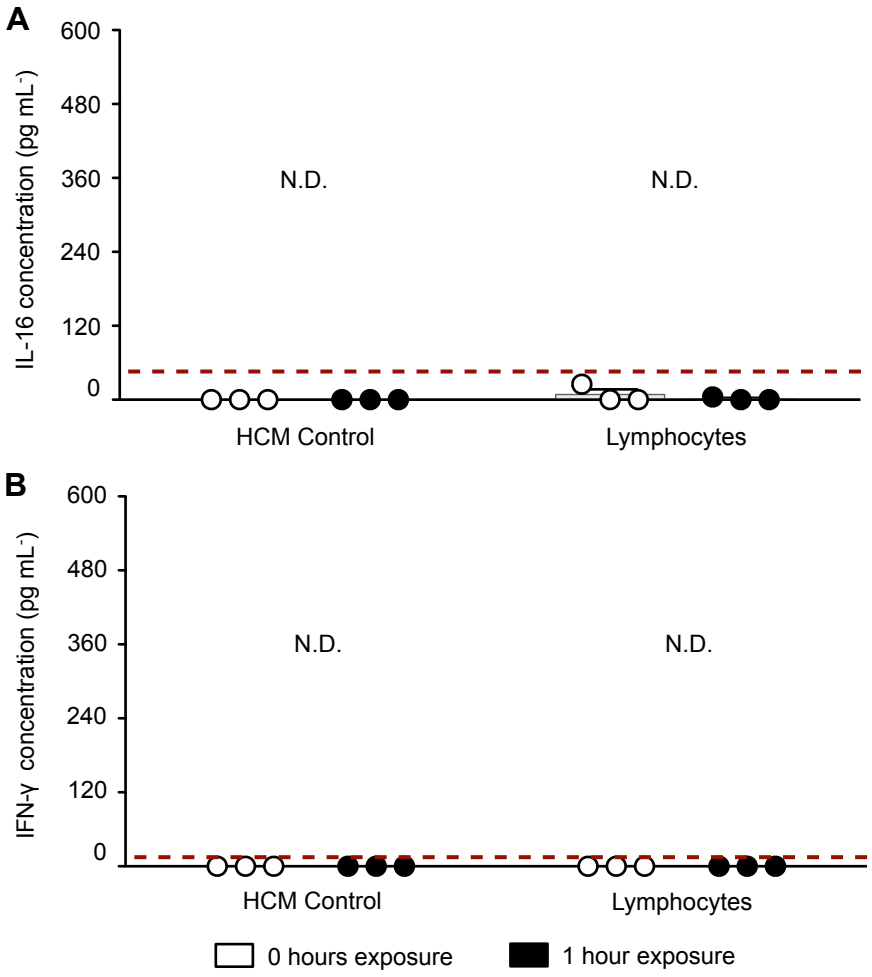

Figure 5.1. IL-16 and IFN-γ are not detectable during lymphocyte exposure of hippocampal cultures. Scatter plots revealing both (A) IL-16 and (B) IFN-γ levels are absent in lymphocyte cell preparations at the start (0 hours exposure) and end of treatment (1-hour exposure). Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in triplicate from three different C57BL/6J hippocampal cultures ($4x10^6$ cells mL^{-1}, n=3). N.D. = non-detectable.

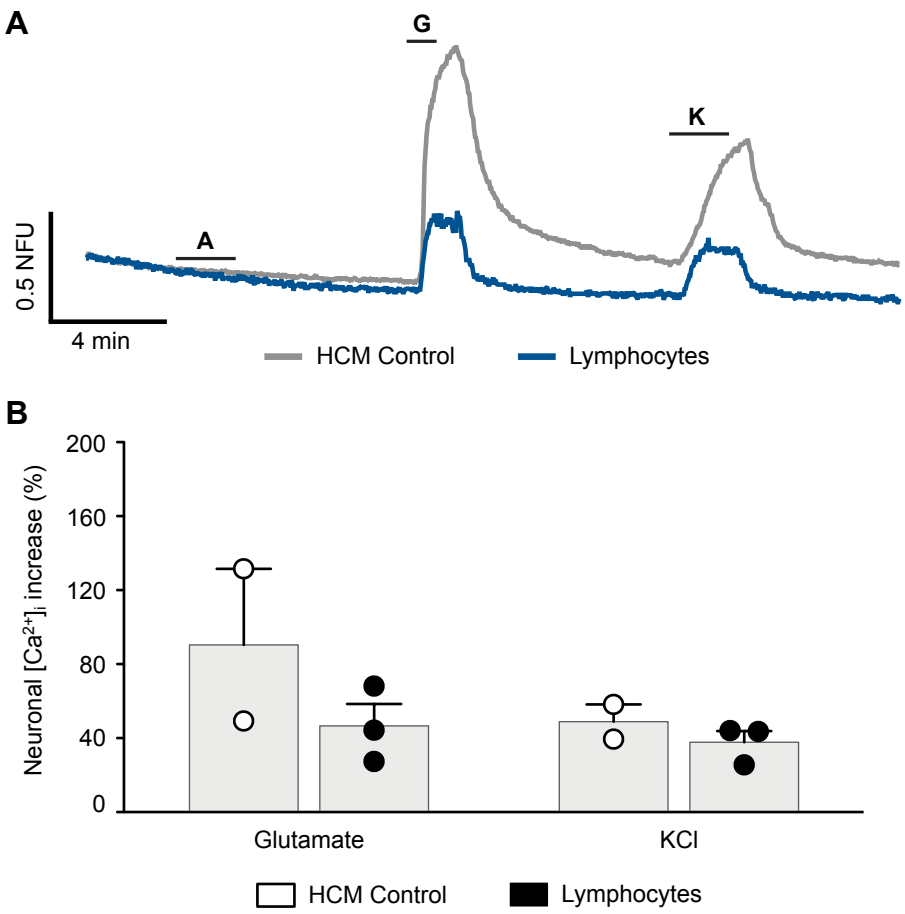

Figure 5.2. Lymphocyte exposure has no effect on glutamate- or KCl-induced neuronal $[Ca^{2+}]_i$ increases. (A) Representative neuronal Ca^{2+} imaging traces of hippocampal cultures exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating no effect of lymphocytes on neuronal glutamate- and KCl-induced $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in triplicate from at least two different C57BL/6J hippocampal cultures (n=2 HCM control treatment and n=3 lymphocyte treatment). A = ADP, G = glutamate, K = KCl and NFU = normalised fluorescent unit.

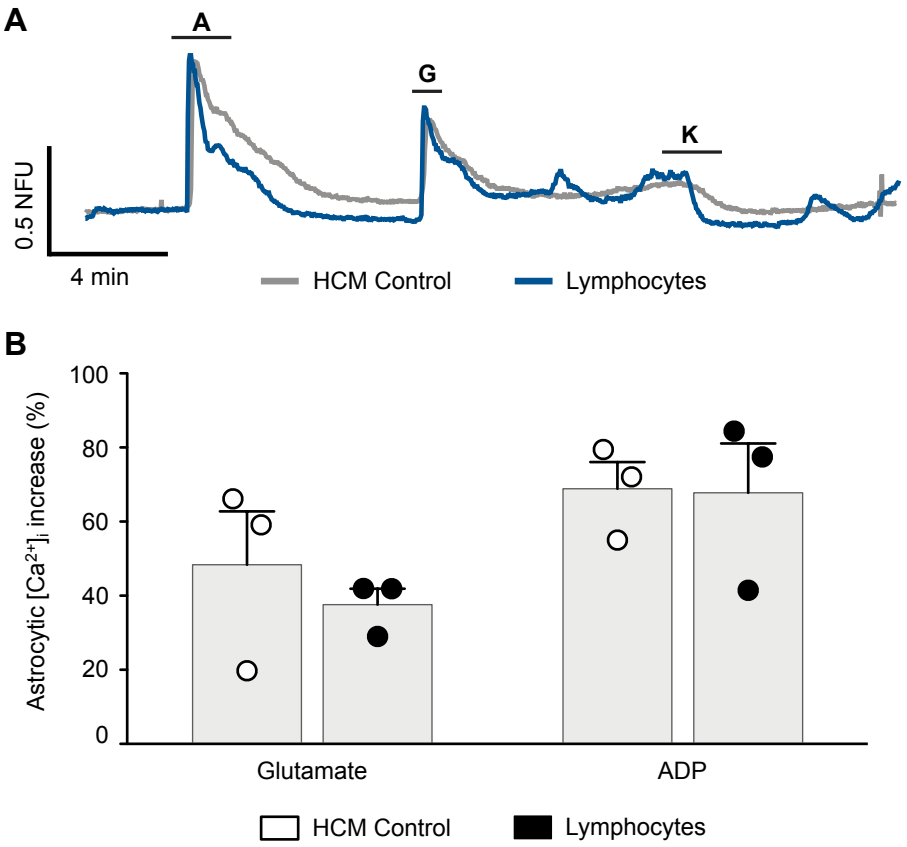

Figure 5.3. Lymphocyte exposure has no effect on glutamate- or ADP-induced astrocytic $[Ca^{2+}]_i$ responses. (A) Representative astrocytic Ca^{2+} imaging traces of hippocampal cultures exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating that lymphocytes have no effect on glutamate- or ADP-induced astrocytic $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in triplicate from at least three different C57BL/6J hippocampal cultures (n=3 for HCM control treatment and n=3 for lymphocyte treatment). A = ADP, G = glutamate, K = KCl and NFU = normalised fluorescent unit.

5.2.3 Hippocampal neurons form synaptic connections between two chambers in microfluidic devices.

Having confirmed that the inflammatory mediators IL-16 and IFN-γ are not present during lymphocyte exposure, and that lymphocytes are without effect on pharmacological-induced

neuronal and astrocytic $[Ca^{2+}]_i$ increases, I investigated the contact-dependent lymphocyte effects on synaptic communication. A microfluidic structure was used to pattern cell growth in order to connect two different hippocampal networks to study synaptic connectivity between them, which was confirmed by immunocytochemistry. Hence, neurite growth was observed in microfluidic chambers and axons (ß-III-tubulin) completely traversed through the microchannels (Figure 5.4). However, dendritic processes (MAP2) did not completely traverse the microfluidic barrier (Figure 5.4). Astrocytic processes (GFAP) were also clearly present within the chambers and initial sections of the microchannels, but these also did not traverse the microchannels (Figure 5.5). In addition, synaptophysin, a presynaptic marker, revealed the presence of synapses both within the chambers and microchannels of the device (Figure 5.6).

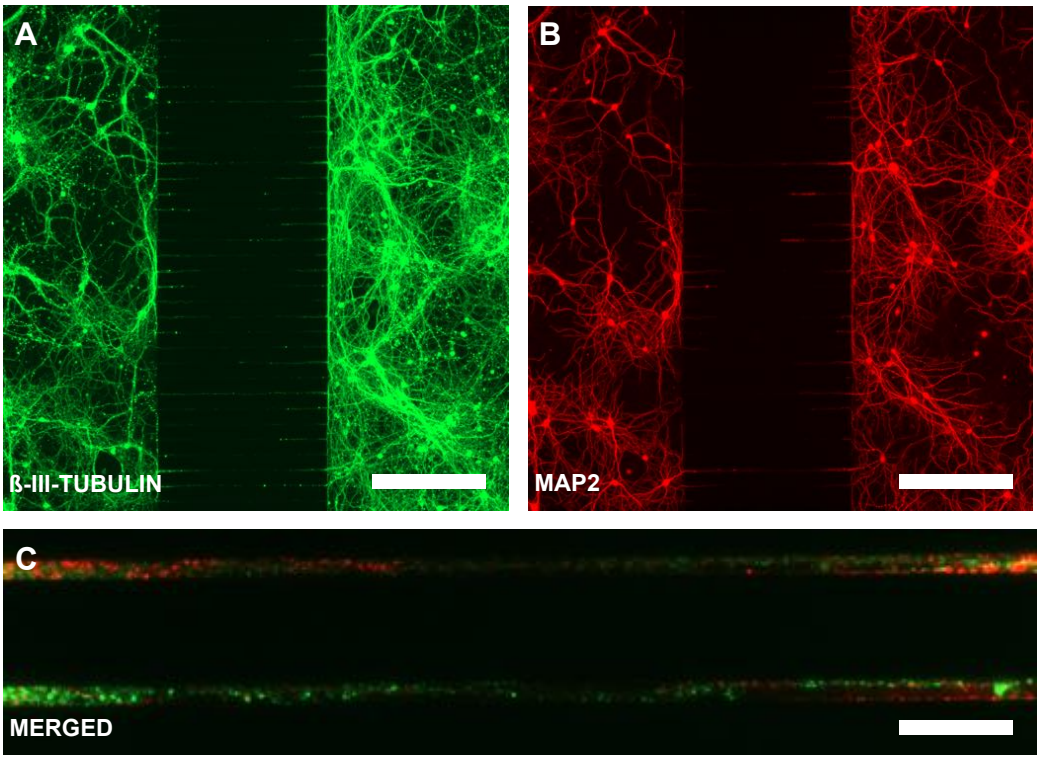

Figure 5.4. Neuronal axons fully traverse across microchannels. (A-C) Representative images showing ß-III-tubulin-positive axons (green), but not MAP2-positive dendrites (red) fully traversing across the microchannels. Data is a representative of two experiments. Scale bar = 100 µm (A & B) and 25 µm (C).

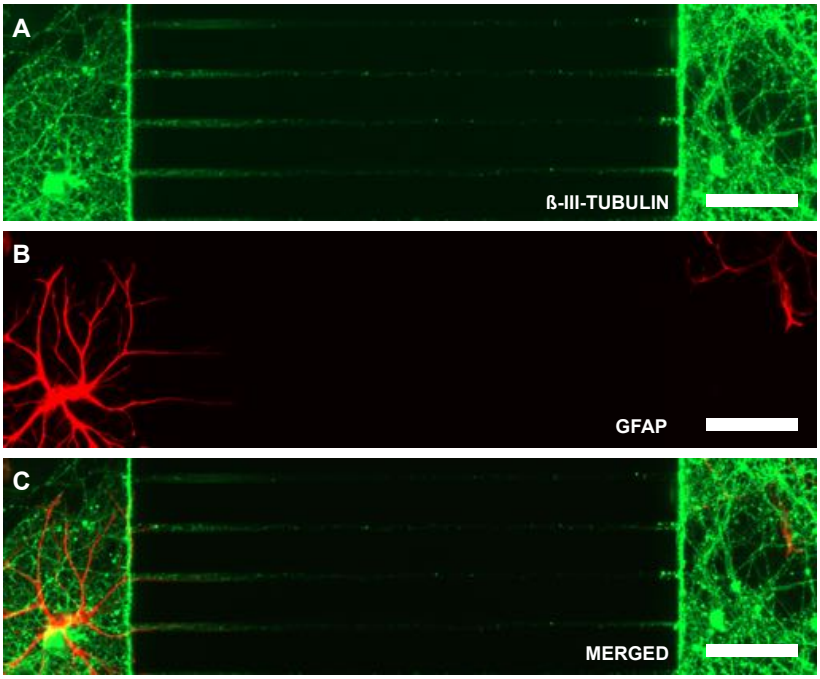

Figure 5.5. Astrocytic processes do not traverse across microchannels. (A-C) Representative images showing ß-III-tubulin-positive axons (green), but not GFAP-positive astrocytes (red) fully traversing across microchannels. Data is a representative of two experiments. Scale bar = 50 µm.

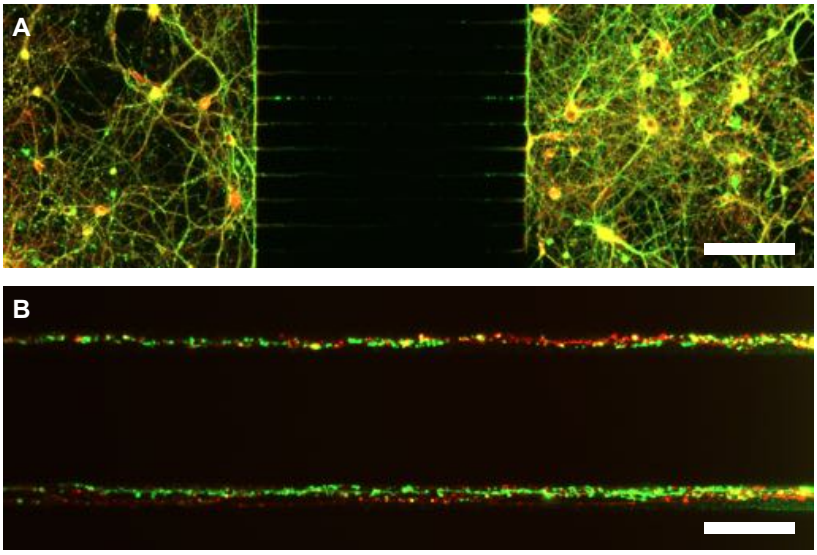

Figure 5.6. Neurons form synaptic connections between two chambers. (A & B) Representative images showing synaptophysin-positive synapse formation (red) with ß-III-tubulin-positive neurons (green) within and outside the microchannels. Data is a representative of two experiments. Scale bar = 100 µm (A) and 16 µm (B).

5.2.4 Creating a hydrostatic pressure gradient between both chambers in microfluidic devices prevents cross contamination of drug delivery.

While the synaptophysin staining strongly suggests that the two hippocampal networks in devices are synaptically connected via neuronal axons, it does not reveal functionality of these connections. Therefore, a live cell assay such as calcium imaging is needed to reveal if one network can activate another network via synaptic communication. To determine this, chemical stimulation of one single network needs to take place by containing the drug into one chamber without cross-contamination into the other chamber, which can be achieved by introducing a hydrostatic pressure gradient. Control experiments were conducted using calcein, a fluorescent marker, to validate drug delivery to a single network without cross-contamination between two chambers. Hence, it was confirmed that calcein was indeed contained within one chamber of a non-cultured microfluidic device following calcein application as previously shown (Figure 5.7B, Robertson et al., 2014, MacKerron et al., 2017). Cross-contamination between two chambers was absent until the hydrostatic pressure gradient was reversed, resulting in the increase in fluorescence in the opposite chamber (Figure 5.7C).

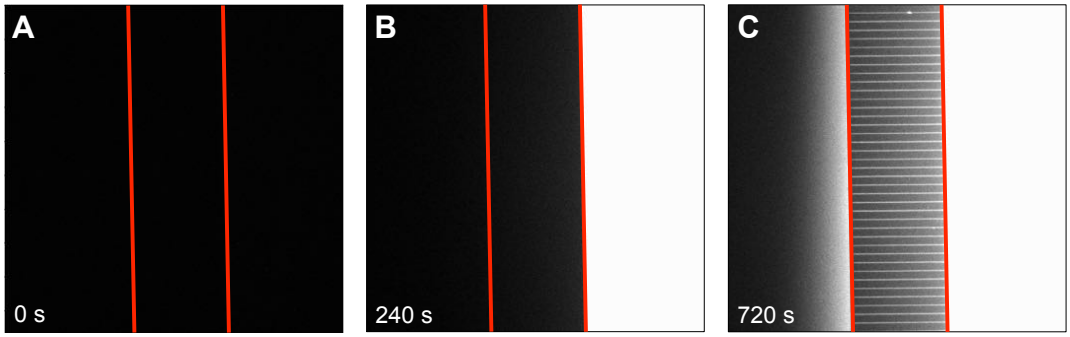

Figure 5.7. A hydrostatic pressure gradient prevents cross-contamination between two chambers of a device. Representative images from (A) experiment start, (B) time point when calcein is applied to the right chamber highlighting no cross-contamination into the left chamber and (C) experiment end when the hydrostatic pressure gradient was reversed and calcein has entered the left chamber via microchannels. Data is a representative of four experiments. Red lines mark microchannel borders with the chambers of a microfluidic device.

5.2.5 Functional neuronal synaptic connectivity exists between two networks present in microfluidic devices.

Having confirmed that drugs can be delivered to a single network in a microfluidic device without cross-contamination to another network, I examined whether the synaptic connections revealed by immunocytochemistry were functional. Hence, cells in the right chamber were stimulated, referred to as the direct chamber (yellow, Figure 5.8A), while output, increases in fluorescence of the calcium dye vs vehicle (Figure 5.8B) was measured in both the right (direct, yellow) and left (indirect, green) chamber (see section 2.3.17).

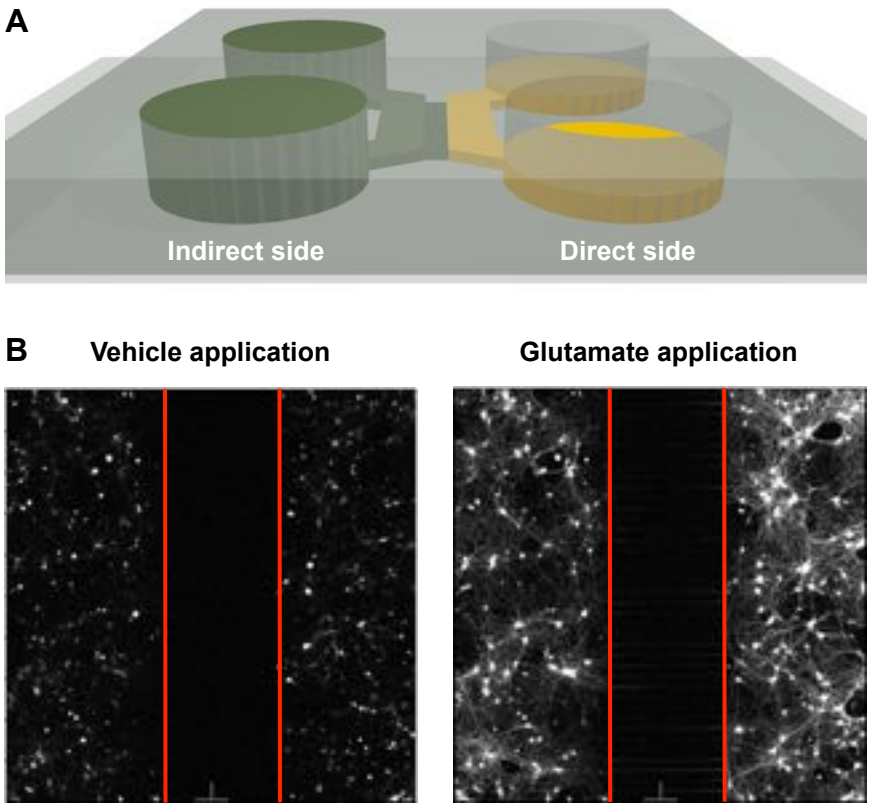

Figure 5.8. Representative images illustrating the experimental setup of calcium imaging in hippocampal cells in microfluidic devices. (A) The direct and indirect side of a microfluidic device during calcium imaging experiments. (B) Fluorescent images of hippocampal cells subjected to vehicle and glutamate application. Red lines mark microchannel borders with the chambers of a microfluidic device.

Glutamate-induced synaptic communication was observed in two out of six devices. Hence, glutamate (10 μM) application in the direct chamber resulted in a significant increase in $n[Ca^{2+}]_i$ levels in the direct chamber (7.7 ± 0.06 NFU, n=2, $p<0.001$ vs baseline, Figure 5.9B) as well as in the indirect chamber (3.5 ± 0.4 NFU, n=2, $p<0.01$ vs baseline, Figure 5.9B). Following KCl (25mM) application in the direct chamber, $n[Ca^{2+}]_i$ levels in the direct chamber were not significantly increased (1.1 ± 0.6 NFU, n=2, Figure 5.9B), but they were in the indirect chamber following KCl application in the indirect chamber (8.1 ± 1.1 NFU, n=2, $p<0.001$ vs baseline, Figure 5.8B). Furthermore, vehicle application in the direct chamber did not elicit any changes in $n[Ca^{2+}]_i$ levels in both the direct and indirect chamber in all experiments (Figure 5.9-5.16). In four out of six devices, glutamate did not significantly induce synaptic communication. While glutamate application in the direct chamber resulted in a significant increase in $n[Ca^{2+}]_i$ levels in the direct chamber (8.5 ± 0.4 NFU, n=3, $p<0.001$ vs baseline, Figure 5.9C), no significant increase in $n[Ca^{2+}]_i$ levels was observed in the indirect chamber (0.2 ± 0.1 NFU, n=3, Figure 5.9C). KCl application in the direct chamber did result in significantly increased $n[Ca^{2+}]_i$ levels in the direct chamber (0.7 ± 0.2 NFU, n=3, $p<0.001$ vs baseline, Figure 5.9C), but not in the indirect chamber (7.6 ± 1.1 NFU, n=3, $p<0.001$ vs baseline, Figure 5.9C) following KCl application in the indirect chamber.

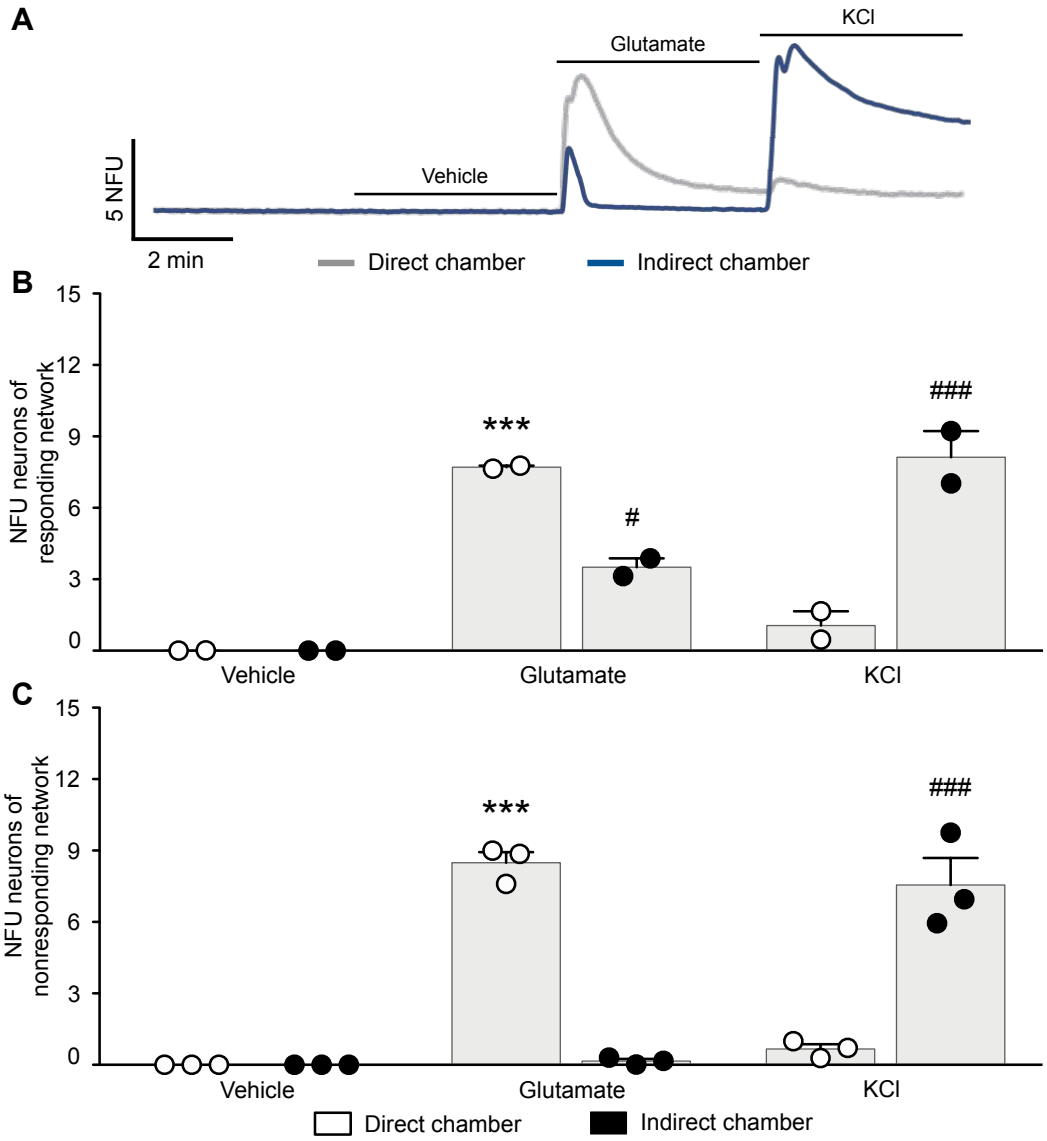

Figure 5.9. Glutamate induces neuronal synaptic communication between two environmentally isolated networks. (A) Representative neuronal Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber of microfluidic devices. (B) Bar chart with scatter plots illustrating that glutamate application in the direct chamber elicits neuronal $[Ca^{2+}]_i$ increases in both the direct and indirect chamber (responding network), (C) but not in all devices (nonresponding network). Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA. *** = $p<0.001$ vs baseline, direct chamber, # = $p<0.01$ and ### = $p<0.001$ vs baseline, indirect chamber. All experiments were carried out from at least two different BALB/c hippocampal cultures (n=2 responding network, n=3 nonresponding network). NFU = normalised fluorescent unit.

Having shown that glutamate application induces neuronal synaptic communication between two networks in microfluidic devices, I investigated the effect of glutamate on astrocyte activity in both networks. In devices in which neuronal synaptic communication was observed, glutamate application in the direct chamber also resulted in a significant increase in a$[Ca^{2+}]_i$ levels in the direct chamber (1.8 ± 0.2 NFU, n=2, $p<0.01$ vs baseline, Figure 5.10B), but not in the indirect chamber (0.2 ± 0.1 NFU, n=2, Figure 5.10B). In contrast to hippocampal astrocytes cultured on coverslips (Figure 5.2), a$[Ca^{2+}]_i$ levels in the direct chamber of microfluidic devices following KCl application in the direct chamber were significantly increased (1.0 ± 0.1 NFU, n=2, $p<0.05$ vs baseline, Figure 5.10B), but not in the indirect chamber (0.9 ± 0.3 NFU, n=2) following KCl application in the indirect chamber. In the devices in which no neuronal synaptic communication was observed, glutamate application in the direct chamber only resulted in a significant increase in a$[Ca^{2+}]_i$ levels in the direct chamber (1.7 ± 0.1 NFU, n=3, $p<0.001$ vs baseline, Figure 5.10C) and not in the indirect chamber (0.2 ± 0.1 NFU, n=3, Figure 5.10C). Furthermore, KCl application in both the direct chamber and indirect chamber significantly increased a$[Ca^{2+}]_i$ levels in the direct chamber (0.9 ± 0.2 NFU, n=3, $p<0.001$ vs baseline, Figure 5.10C) and the indirect chamber (1.6 ± 0.2 NFU, n=3, $p<0.001$ vs baseline, Figure 5.10C), respectively.

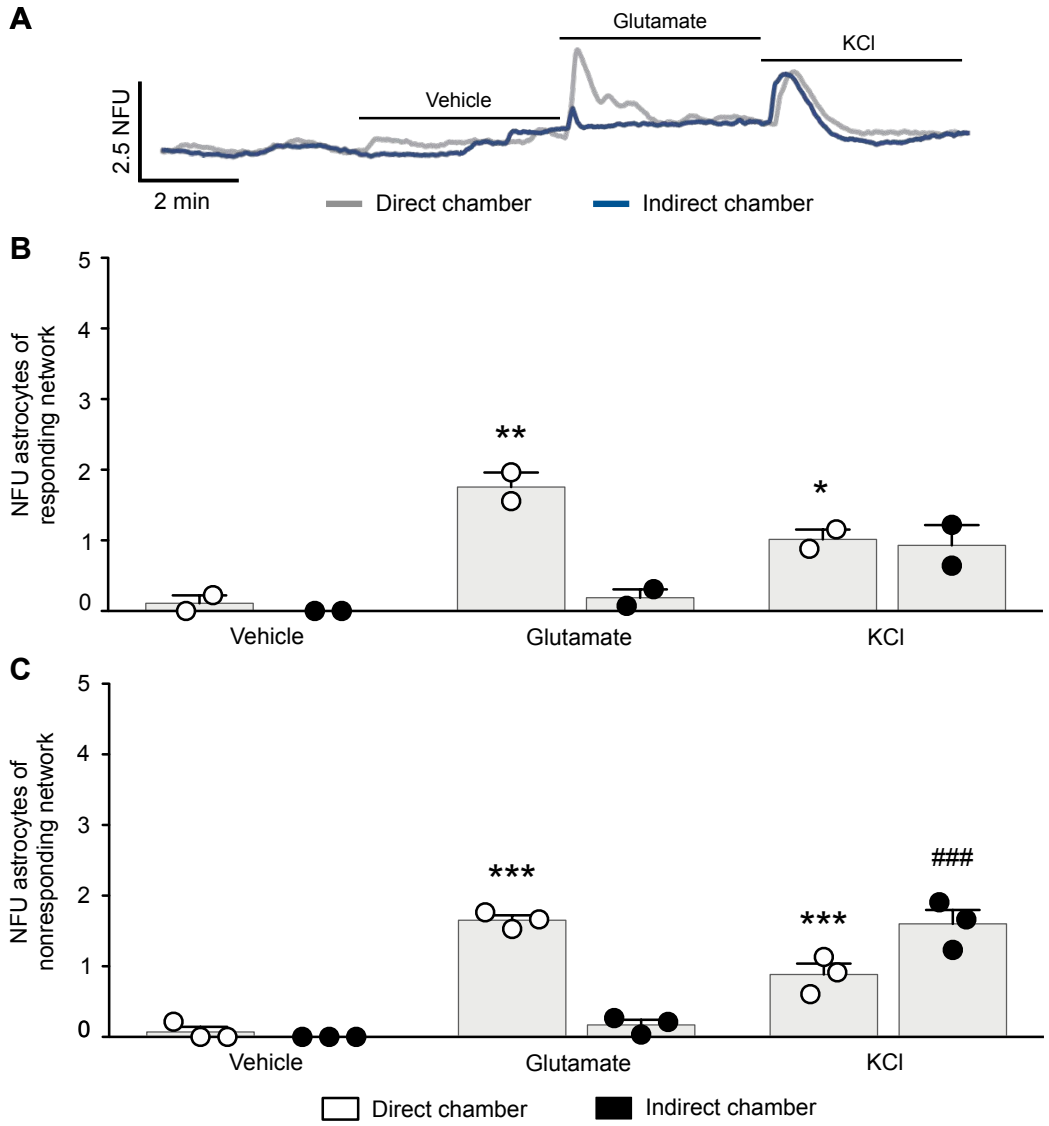

Figure 5.10. Glutamate application in the direct chamber induces astrocytic $[Ca^{2+}]_i$ increases. (A) Representative astrocytic Ca^{2+} imaging traces of hippocampal cells cultured in direct and indirect chamber of microfluidic devices. (B) Bar chart with scatter plots illustrating that glutamate application in the direct chamber elicits astrocytic $[Ca^{2+}]_i$ increases in both the direct and indirect chamber (responding network), (C) but not in all devices (nonresponding network). Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ vs baseline, direct chamber, ### = $p<0.001$ vs baseline, indirect chamber. All experiments were carried out from three different BALB/c hippocampal cultures (n=3 responding network, n=3 nonresponding network). NFU = normalised fluorescent unit.

5.2.6 Lymphocytes do not induce hippocampal cell death.

Having shown that glutamate induces functional neuronal synaptic communication between hippocampal networks in microfluidic devices, I investigated whether lymphocytes cause hippocampal cell death in microfluidic devices. Hence, live/dead stainings revealed that cell survival following lymphocyte exposure in the direct chamber (79.2 ± 4.7%, n=3, Figure 5.11) was not significantly different from cell survival following HCM control exposure in the direct chamber (82.6 ± 1.9%, n=3, Figure 5.11). Furthermore, no difference was observed between cell survival in the indirect chamber following HCM exposure in the indirect chamber and lymphocyte exposure in the direct chamber (77.6 ± 5.3%, n=3, Figure 5.11) and HCM exposure in the indirect chamber following HCM exposure in both the direct and indirect chamber (79.1 ± 0.2%, n=3, Figure 5.11). In addition, no difference was observed between cell survival following lymphocyte exposure in the direct chamber compared to HCM exposure in the indirect chamber of the same device.

5.2.7 HCM and lymphocyte exposure prevent synaptic communication.

Having shown that lymphocytes are not toxic to hippocampal cells, I investigated contact-dependent effects of lymphocytes on hippocampal connected networks. In agreement with previous experiments on coverslips (Figure 5.1), lymphocyte exposure (1 hour) in the direct chamber has no effect on glutamate-induced n$[Ca^{2+}]_i$ increases (4.3 ± 0.8 NFU, n=3, Figure 5.12B) compared to HCM control (3.7 NFU ± 0.5, n=5, Figure 5.12B). In contrast to previous experiments in external solution (Figure 5.9), following HCM control and lymphocyte exposure, glutamate application in the direct chamber did not elicit any changes in n$[Ca^{2+}]_i$ levels in the indirect chamber (HCM control: 0.02 ± 0.01 NFU, n=5, lymphocytes: 0.05 ± 0.03 NFU, n=3, Figure 5.12C). KCl-induced increases in n$[Ca^{2+}]_i$ in both the direct and indirect chamber following lymphocyte exposure (direct: 0.4 ± 0.2 NFU, indirect: 3.5 ± 0.4 NFU, n=3, Figure 5.12B & C) were not significantly different from HCM control (direct: 0.4 ± 0.1 NFU, indirect: 3.2 ± 0.4 NFU, n=5, Figure 5.12B & C).

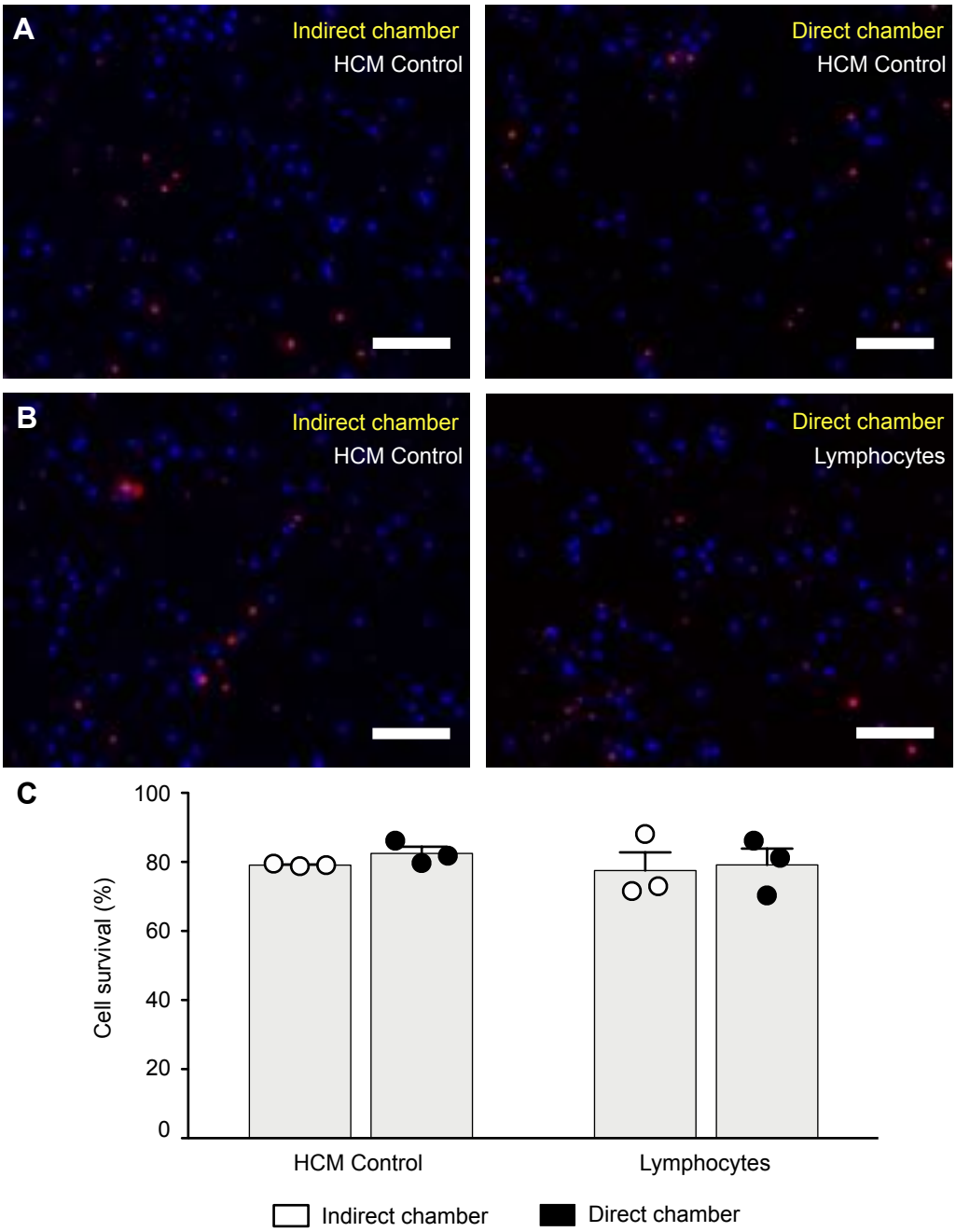

Figure 5.11. Lymphocytes are not toxic to hippocampal cells. (A & B) Representative images of hippocampal cultures stained with PI (red) and Hoechst (blue) following exposure to HCM control or lymphocytes in direct and indirect chamber. (C) Bar chart with scatter plots illustrating that lymphocytes do not affect hippocampal viability. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA with a Bonferroni's post-hoc comparison. All experiments were carried out in triplicate from three different BALB/c hippocampal cultures (n=3). Scale bar = 160 µm.

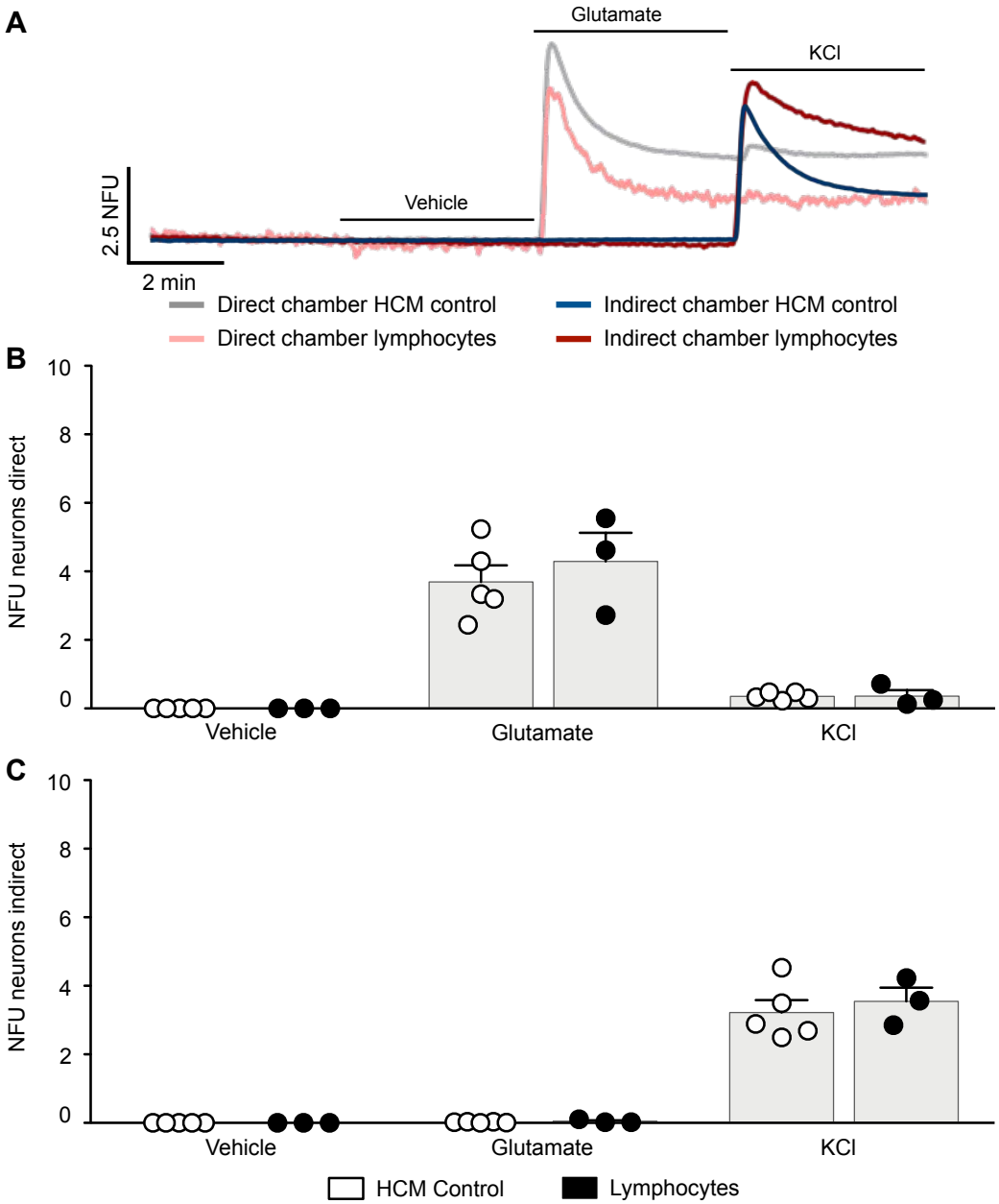

Figure 5.12. Lymphocyte exposure has no effect on glutamate-induced n[Ca^{2+}]$_i$ increases in the direct chamber. (A) Representative neuronal Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating no effect of lymphocytes on glutamate-induced neuronal [Ca^{2+}]$_i$ increases and (C) prevention of synaptic communication similar to HCM control. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out from at least three different BALB/c hippocampal cultures (n=5 HCM control treatment and n=3 lymphocyte treatment). NFU = normalised fluorescent unit.

Lymphocyte exposure of hippocampal cultures in microfluidic devices (1 hour) has no effect on glutamate- or KCl-induced a$[Ca^{2+}]_i$ increases in the direct chamber (glutamate:1.4 ± 0.4 NFU, KCl: 0.8 ± 0.1 NFU, n=3, Figure 5.13B) compared to HCM control (glutamate: 1.3 ± 0.3 NFU, KCl: 0.8 ± 0.1 NFU, n=5, Figure 5.13B). Following both HCM control and lymphocyte exposure, glutamate application in the direct chamber did not elicit any changes in a$[Ca^{2+}]_i$ levels in the indirect chamber (HCM control: 0.02 ± 0.01 NFU, n=5, lymphocytes: 0.04 ± 0.01 NFU, n=3, Figure 5.13C). Furthermore, KCl-induced a$[Ca^{2+}]_i$ increases in the indirect chamber following lymphocyte exposure (1.4 ± 0.4 NFU, n=3, Figure 5.13C) were not significantly different from HCM control (1.31 ± 0.1 NFU, n=5, Figure 5.13C).

5.2.8 ADP application increases astrocytic intracellular calcium levels in the direct chamber but not in the indirect chamber.

Given that ADP is an astrocyte specific agonist and does not induce $[Ca^{2+}]_i$ increases in neurons (Figure 4.2 and 5.1), I used ADP to specifically elicit $[Ca^{2+}]_i$ increases in astrocytes and thereby investigate their activity in microfluidic devices. Hence, ADP application in the direct chamber resulted in significant increases in a$[Ca^{2+}]_i$ levels in the direct chamber (2.0 ± 0.3 NFU, n=3, $p<0.001$ vs baseline, Figure 5.14) but not in the indirect chamber (0.1 ± 0.02 NFU, n=3, Figure 5.14). Interestingly, KCl application in the direct and indirect chamber elicited significant increases in a$[Ca^{2+}]_i$ in the direct (0.9 ± 0.1 NFU, n=3, $p<0.05$ vs baseline, Figure 5.14) and indirect chamber (1.8 ± 0.2 NFU, n=3, Figure 5.14), respectively.

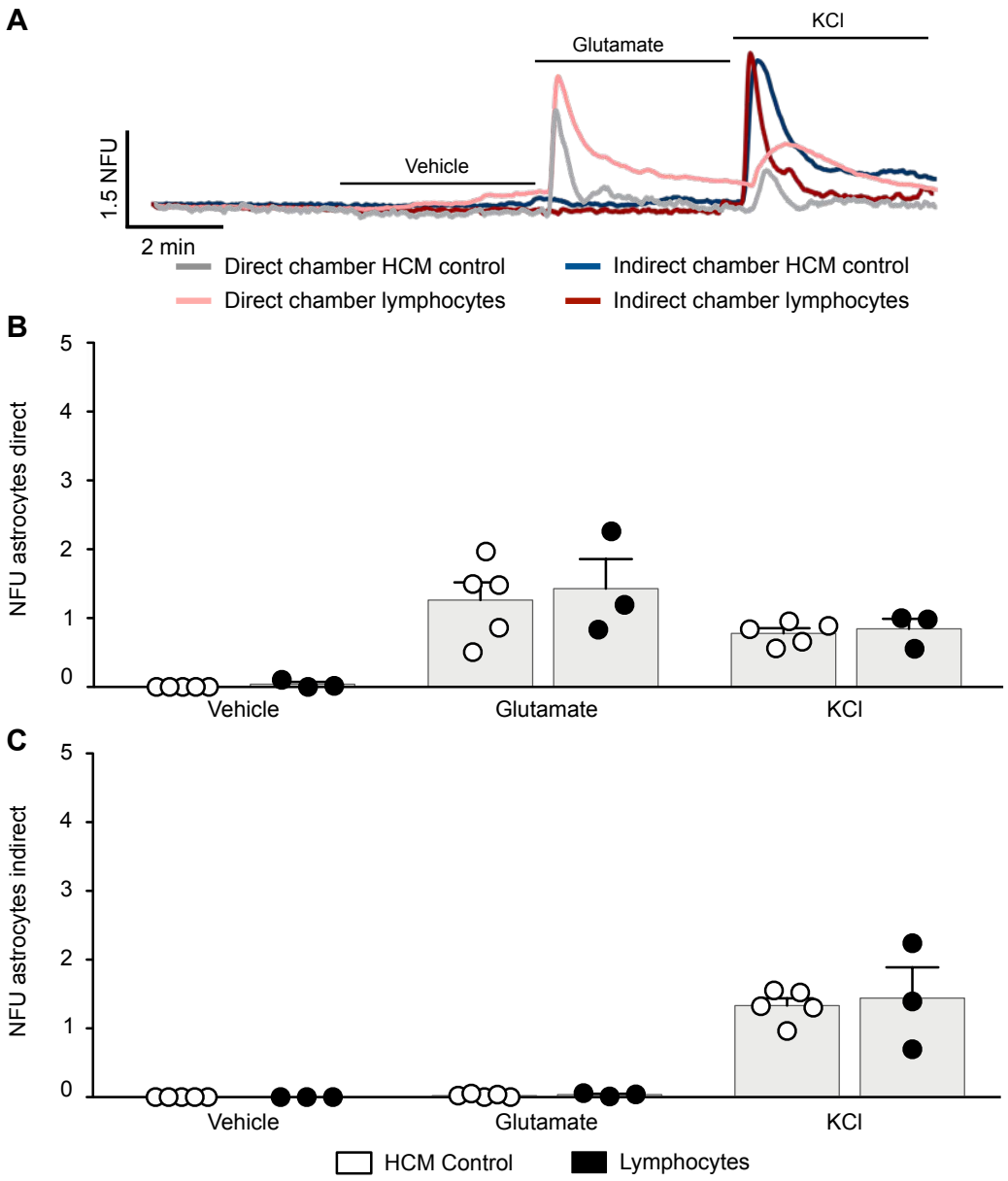

Figure 5.13. Lymphocyte exposure has no effect on glutamate-induced astrocytic $[Ca^{2+}]_i$ increases in the direct chamber. (A) Representative astrocytic Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber, exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating no effect of lymphocytes on glutamate- and KCl-induced astrocytic $[Ca^{2+}]_i$ increases in the direct and (C) indirect chamber. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in at least three different BALB/c hippocampal cultures (n=5 HCM control treatment and n=3 lymphocyte treatment). NFU = normalised fluorescent unit.

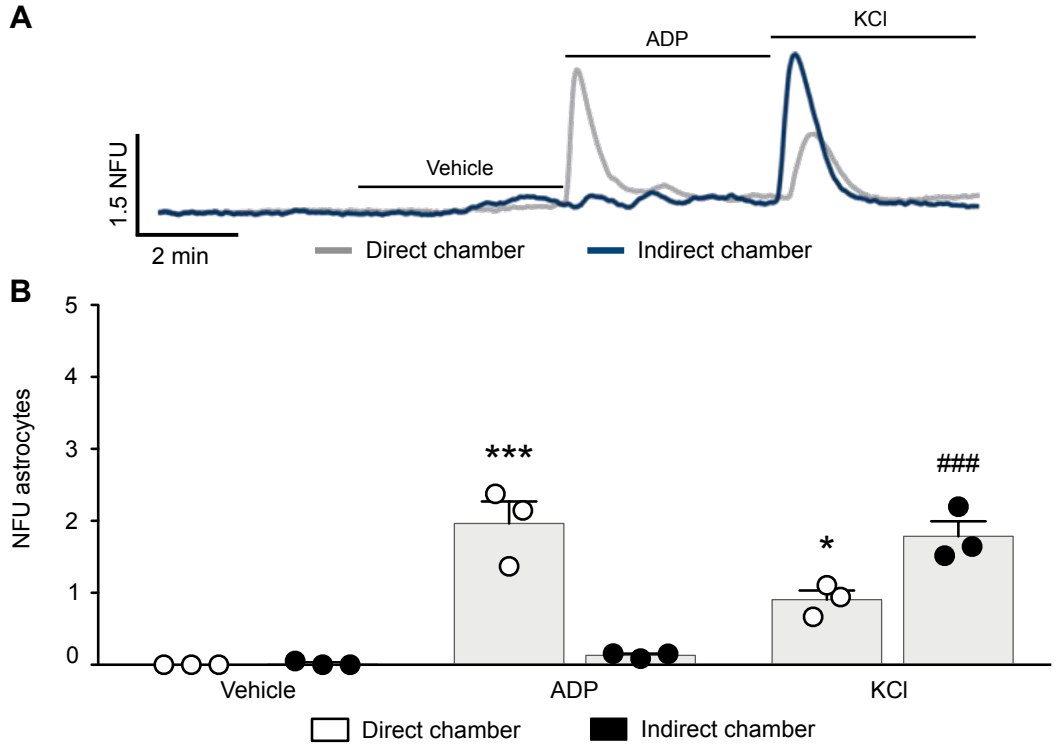

Figure 5.14. ADP application induces increases in astrocytic $[Ca^{2+}]_i$ in the direct chamber. (A) Representative astrocytic Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber. (B) Bar chart with scatter plots illustrating that ADP application in the direct chamber elicits astrocytic $[Ca^{2+}]_i$ increases in the direct but not indirect chamber. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA. * = $p<0.05$ and *** = $p<0.001$ vs baseline, direct chamber and ### = $p<0.001$ vs baseline, indirect chamber. All experiments were carried out in duplicate from three different BALB/c hippocampal cultures (n=3). NFU = normalised fluorescent unit.

In contrast to astrocytes but in agreement with previous experiments on coverslips (Figure 4.2 and 5.1), following ADP application in the direct chamber, no significant increase in $n[Ca^{2+}]_i$ levels was observed in the direct (0.03 ± 0.02 NFU, n=3, Figure 5.15) and indirect chamber (0.0 ± 0.0 NFU, n=3, Figure 5.15). Neuronal $[Ca^{2+}]_i$ levels were significantly increased in

response to direct KCl application in the direct (7.9 ± 0.8 NFU, n=3, p<0.001 vs baseline, Figure 5.15) and indirect chamber (8.8 ± 1.3 NFU, n=3, p<0.001 vs baseline, Figure 5.15).

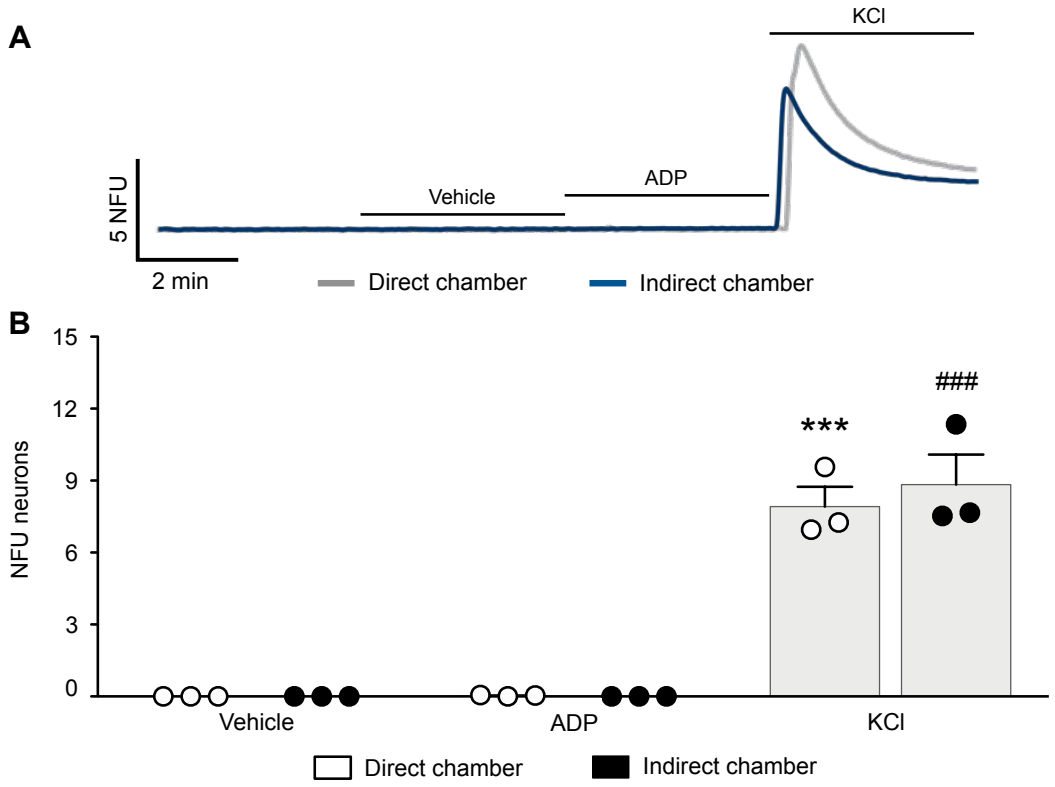

Figure 5.15. ADP application does not elicit any changes in neuronal $[Ca^{2+}]_i$ levels. (A) Representative neuronal Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber of microfluidic devices. (B) Bar chart with scatter plots illustrating that ADP application in the direct chamber has no effect on neuronal $[Ca^{2+}]_i$ increases in both the direct and indirect chamber. KCl application in both the direct and indirect chamber induces neuronal $[Ca^{2+}]_i$ increases. Data are presented as mean ± S.E.M. and analysed using a one-way ANOVA. *** = p<0.001 vs baseline for the direct chamber and ### = p<0.001 vs baseline for the indirect chamber. All experiments were carried out in duplicate from three different BALB/c hippocampal cultures (n=3). NFU = normalised fluorescent unit.

5.2.9 Lymphocyte exposure has no effect on ADP-induced intracellular calcium increases.

In agreement with previous experiments on coverslips (Figure 5.2), lymphocyte exposure to hippocampal cultures in microfluidic devices (1 hour) did not significantly impair ADP-induced a$[Ca^{2+}]_i$ increases in the direct chamber (1.7 ± 0.3 NFU, n=3, Figure 5.16B) compared to HCM control (2.2 ± 0.1 NFU, n=3, Figure 5.16B). Following both HCM control and lymphocyte exposure, no change was observed in a$[Ca^{2+}]_i$ levels in the indirect chamber when ADP was applied in the direct chamber (HCM: 0.02 ± 0.01 NFU, lymphocytes: 0.02 ± 0.02 NFU, n=3, Figure 5.16C). Hence, lymphocyte exposure was without effect on a$[Ca^{2+}]_i$ increases in the indirect chamber when ADP was applied in the direct chamber. Furthermore, lymphocyte exposure (1 hour) had no effect on KCl-induced a$[Ca^{2+}]_i$ increases in the direct (0.7 ± 0.1 NFU, n=3, Figure 5.16B) and indirect chamber (1.4 ± 0.4 NFU, n=3, Figure 5.16C) when compared to HCM control (direct: 0.5 ± 0.02 NFU, indirect: 1.4 ± 0.04 NFU, n=3, Figure 5.16B and C).

Following both HCM control and lymphocyte exposure, no change was observed in n$[Ca^{2+}]_i$ levels in the direct chamber (HCM: 0.1 ± 0.04 NFU, lymphocytes: 0.1 ± 0.01 NFU, n=3, Figure 5.17B) as well as the indirect chamber (HCM: 0.004 ± 0.004 NFU, lymphocytes: 0.002 ± 0.002 NFU, n=3, Figure 5.17C) when ADP was applied in the direct chamber. Lymphocyte exposure had no effect on KCl-induced n$[Ca^{2+}]_i$ increases in both the direct chamber (5.4 ± 0.2 NFU, n=3, Figure 5.17B) and indirect chamber (4.1 ± 0.3 NFU, n=3, Figure5.17C) when compared to HCM control (direct: 4.4 ± 0.4 NFU, indirect: 3.0 ± 0.6 NFU, n=3, Figure 5.17A and B).

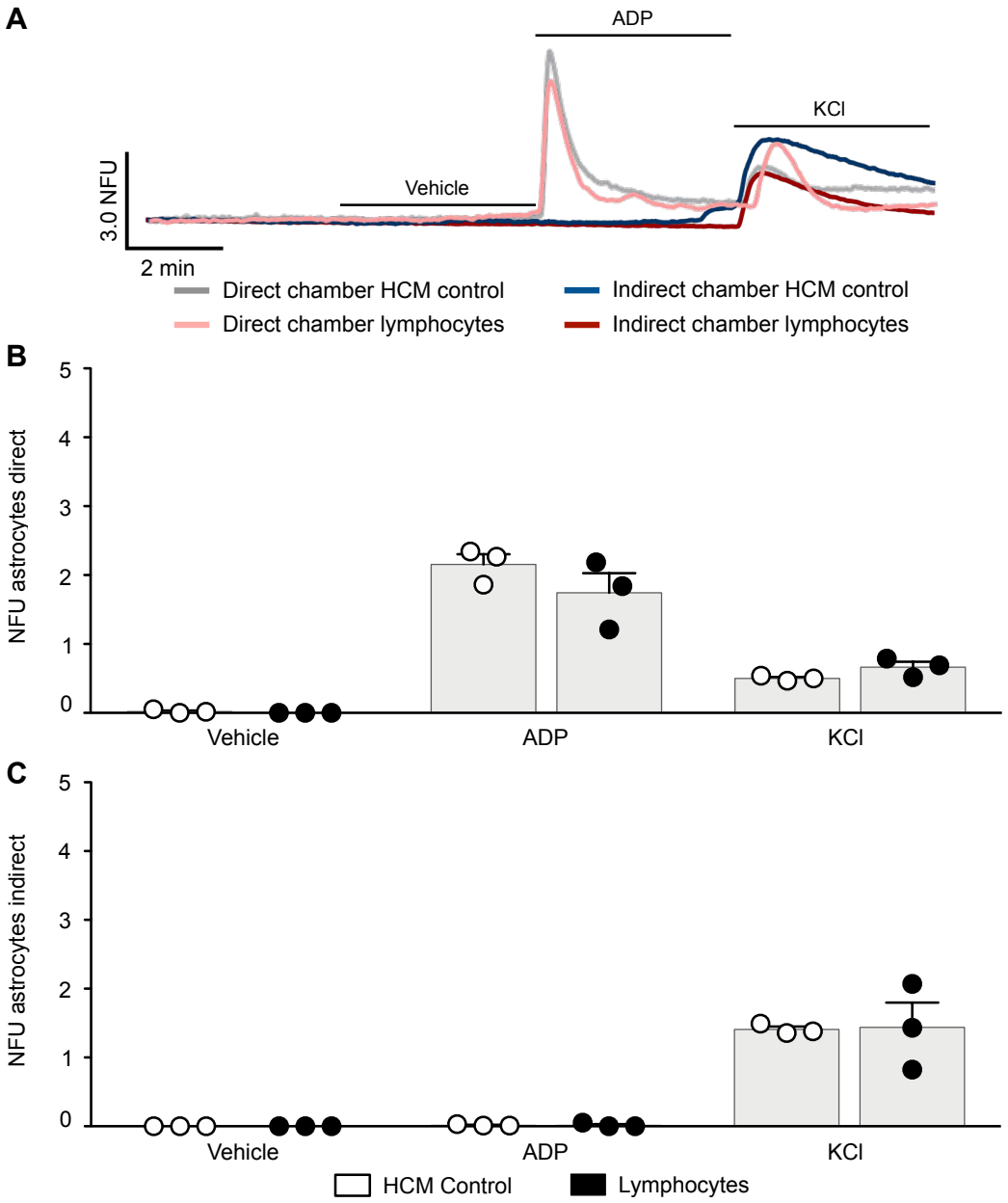

Figure 5.16. Lymphocyte exposure has no effect on ADP-induced astrocytic $[Ca^{2+}]_i$ increases. (A) Representative astrocytic Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber, exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating no effect of lymphocytes on ADP-induced astrocytic $[Ca^{2+}]_i$ increases in the direct or (C) indirect chamber. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in duplicate from at least three different BALB/c hippocampal cultures (n=3). NFU = normalised fluorescent unit.

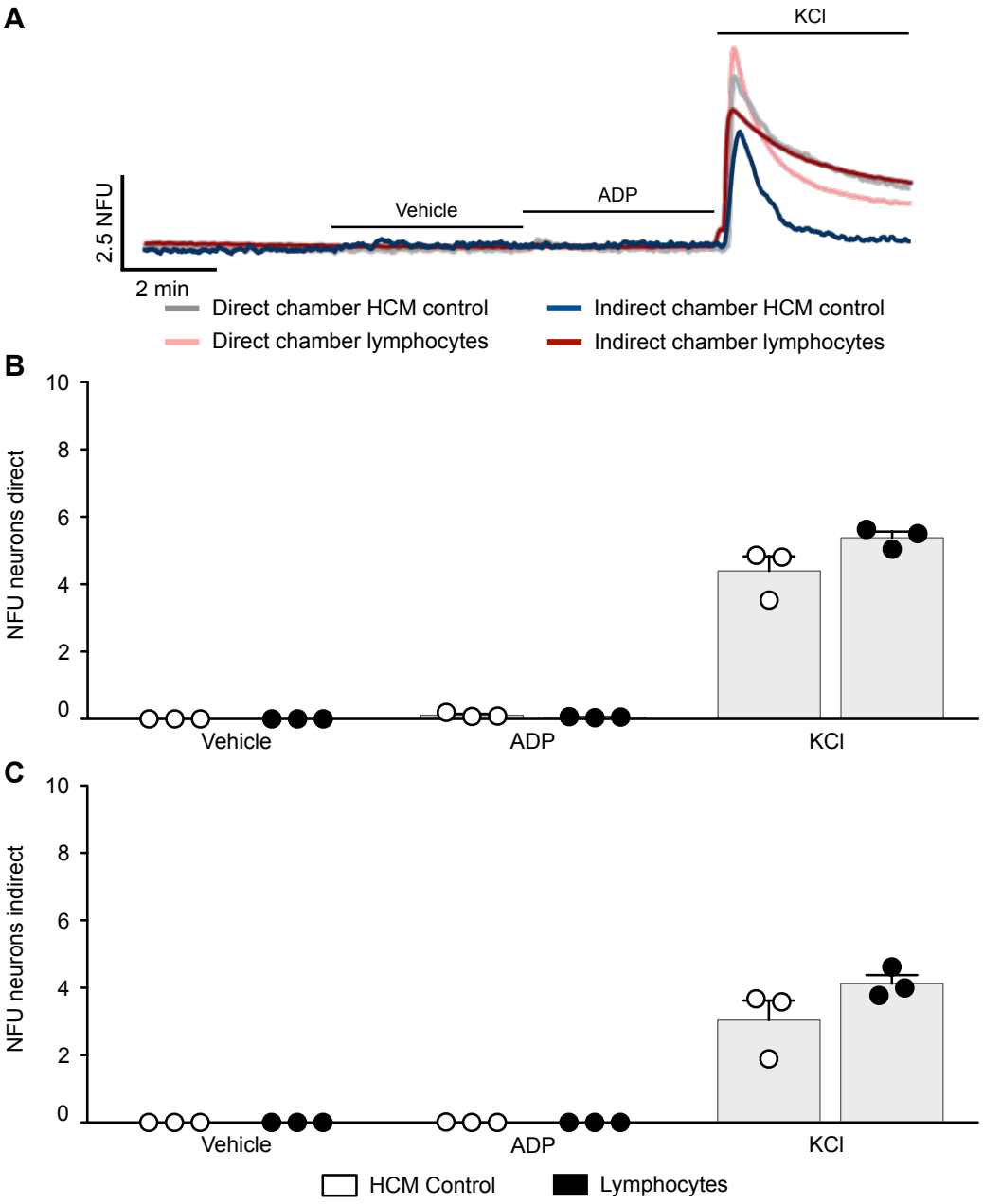

Figure 5.17. Lymphocyte exposure has no effect on ADP-induced neuronal $[Ca^{2+}]_i$ increases in both chambers. (A) Representative neuronal Ca^{2+} imaging traces of hippocampal cells cultured in the direct and indirect chamber, exposed to HCM control or lymphocytes. (B) Bar chart with scatter plots illustrating no effect of lymphocytes on ADP-induced neuronal $[Ca^{2+}]_i$ increases in the direct or (C) indirect chamber. Data are presented as mean ± S.E.M. and analysed using an unpaired Student's t-test. All experiments were carried out in duplicate from three different BALB/c hippocampal cultures (n=3). NFU = normalised fluorescent unit.

5.2.10 Lymphocytes do not migrate towards CCL19 in microfluidic devices.

In the second part of this chapter, I intended to examine whether lymphocyte migratory behaviour changes following induction of hippocampal cell death or increased neuronal activity. Therefore, I first investigated whether it is possible to study lymphocyte migration in microfluidic devices by adding a well-known lymphocyte chemoattractant (CCL19) to the direct chamber whilst lymphocytes were loaded in the indirect chamber. I tested two different coatings, PLO as used for hippocampal cultures and collagen. Lymphocytes were clearly present in the indirect chamber in PLO-coated devices (Figure 5.18) but using a collagen coating resulted in a larger number present than within PLO-coated devices (Figure 5.19). Nevertheless, lymphocytes cultured on both coatings showed no propensity to migrate towards the direct chamber containing CCL19 (100 ng mL^{-1}, Figure 5.18 and 5.19). Hence, lymphocytes did not gather near the microchannels at all tested time points, with the exception of one collagen-coated device (Figure 5.19D). To test whether this observed lymphocyte migration was indeed mediated by the chemoattractant CCL19, I introduced a small hydrostatic pressure gradient in favour of CCL19 (100 ng mL^{-1}), which drives CLL19 into the direction of the lymphocytes. However, lymphocytes showed no propensity to migrate towards the direct chamber or gather near the microchannels in order to migrate through them (Figure 5.20A). Interestingly, following microfluidic device tilting, which creates a small fluid flow towards the direct chamber containing CCL19 (100 ng mL^{-1}), lymphocytes were pushed towards this chamber and gathered near microchannels, irrespective of CCL19 presence (Figure 5.20B). This suggested that I was not able to study chemoattractant-driven lymphocyte migration in microfluidic devices under my experimental conditions.

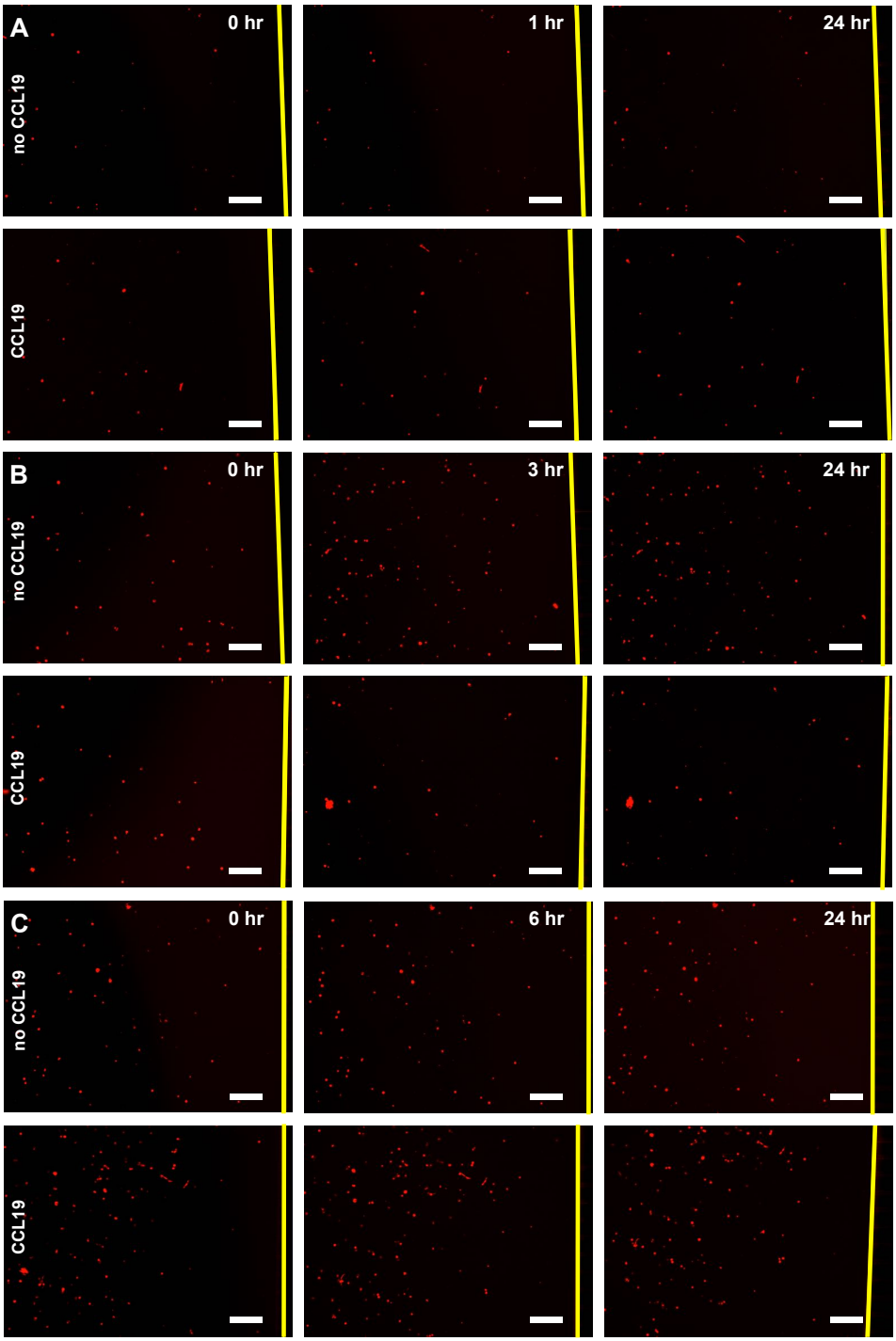

Figure 5.18. Lymphocytes do not migrate towards a CCL19-containing chamber in PLO-coated devices. Representative images of lymphocytes (red) present in the indirect chamber of PLO-coated devices in the presence or absence of CCL19 in the direct chamber for (A) 1-24, (B) 3-24 and (C) 6-24 hours. Data is a representative of two experiments. Yellow lines mark microchannel borders with the indirect chamber. Scale bar = 50 μm.

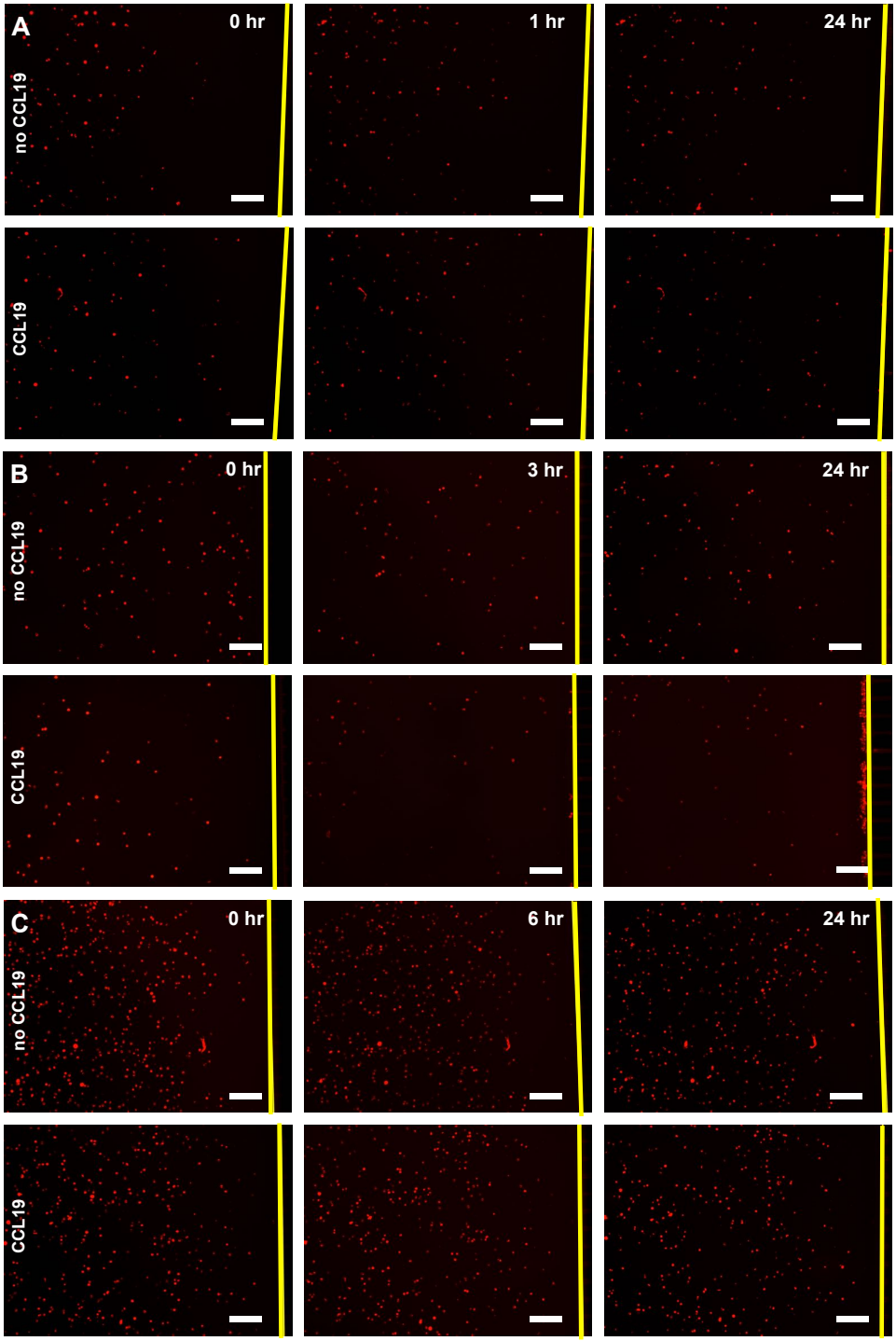

Figure 5.19. Lymphocytes do not migrate towards a CCL19-containing chamber in collagen-coated devices. Representative images of lymphocytes (red) present in the indirect chamber of PLO-coated devices in the presence or absence of CCL19 in the direct chamber for (A) 1-24, (B) 3-24 and (C) 6-24 hours. Data is a representative of two experiments. Yellow lines mark microchannel borders with indirect chamber. Scale bar = 50 µm.

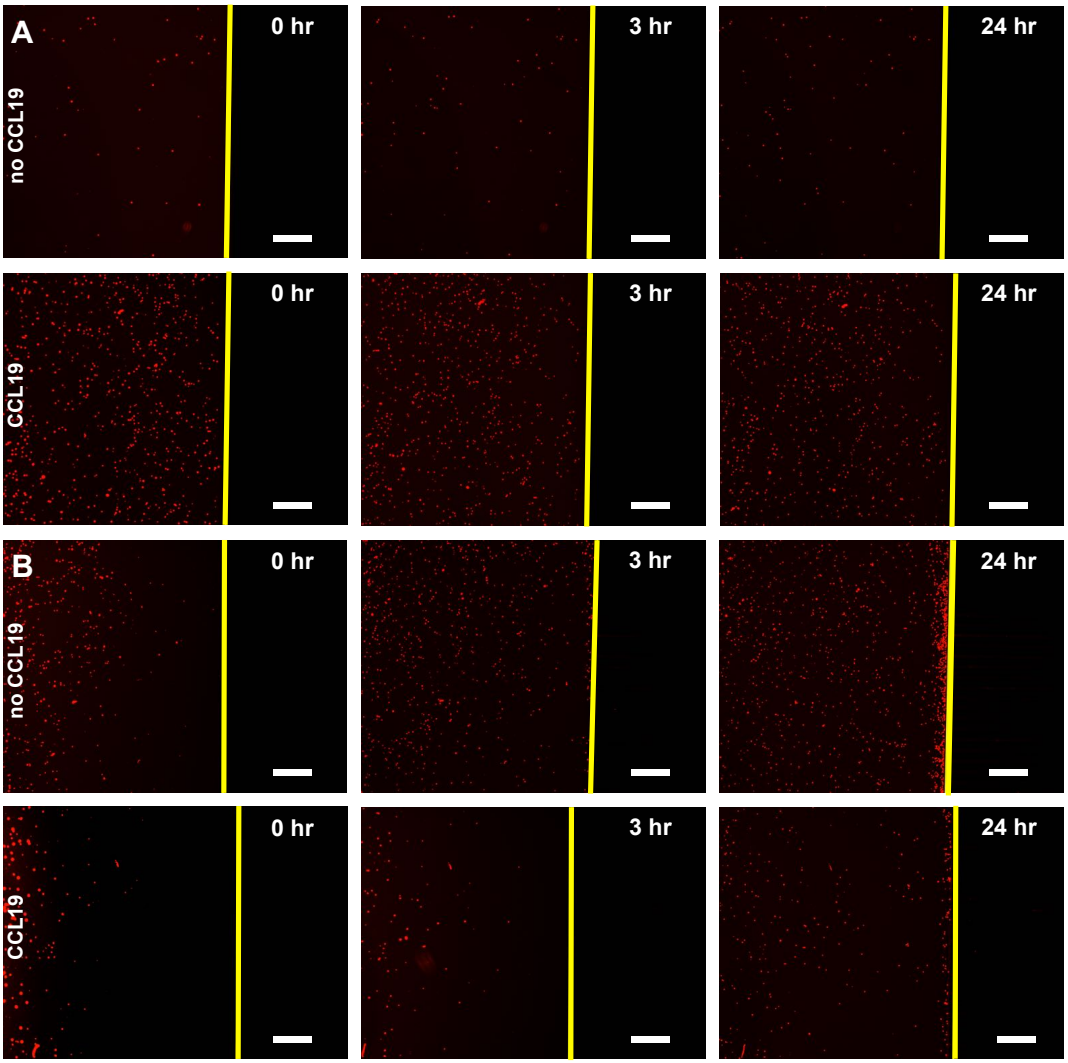

Figure 5.20. Lymphocytes gather near microchannels due to device tilting. Representative images of lymphocytes (red) present in the indirect chamber of a collagen-coated device, in the presence or absence of CCL19 in the direct chamber. (A) A hydrostatic pressure gradient in favour of the direct chamber containing CCL19 did not induce lymphocyte migration. (B) In tilted devices, introducing a fluid flow from left to right, lymphocytes gathered near microchannels, irrespective of CCL19. Data is a representative of one experiment. Yellow lines mark microchannel borders with the indirect chamber. Scale bar = 50 µm.

5.3 Discussion

To better understand the functional role of lymphocytes in the CNS, I exposed hippocampal cultures to naïve lymphocytes. My data reveals that lymphocytes have no effect on pharmacological-induced $[Ca^{2+}]_i$ increases in neurons cultured on coverslips. These results were confirmed in subsequent microfluidic devices. Furthermore, I have shown functional glutamate-induced synaptic communication between two mouse hippocampal networks, but the effect of lymphocytes on synaptic communication could not be investigated.

5.3.1 Naïve lymphocytes have no toxic effects on hippocampal cells.

Whilst most CNS studies focus on immune cells in their effector or effector-memory state, naïve immune cell infiltration into the CNS has also been shown (Cose *et al.*, 2006; Herz *et al.*, 2011). However, the role of naïve lymphocytes in either CNS homeostasis or disease states is still unclear. It was shown though that whereas activated T cells induce cell death in human fetal neurons in a contact-dependent manner, naïve $CD4^+$ and $CD8^+$ T cells did not (Giuliani *et al*., 2003). Furthermore, naïve $CD4^+CD25+Foxp3+$ T cells are not neurotoxic to human neurons and thus remain healthy when co-cultured (Haile *et al.*, 2011). Hence, before naïve lymphocytes were co-cultured with hippocampal cells under my experimental conditions, I examined whether naïve lymphocytes induce neurotoxicity. My data shows that cell survival following lymphocyte exposure is similar to HCM control exposure in the direct chamber. Cell survival following HCM control exposure is similar to a previous study in our laboratory which showed a $73.8 \pm 0.01\%$ cell survival using live/dead imaging carried out on microfluidic cultures immediately after being removed from the incubator (MacKerron *et al*., 2017). Similar to cells in the direct chamber exposed to lymphocytes, hippocampal cells in the indirect chamber who were exposed to HCM control were also unaffected, indicating that lymphocytes did not spread any effects to the other connected chamber. Hence, I show that naïve lymphocytes do not induce neurotoxicity, which is in agreement with what is found in the literature (Giuliani et al., 2003, Nitsch et al., 2004, Haile *et al*., 2011, Haile *et al*., 2015).

5.3.2 Lymphocytes have no effects on pharmacological-induced intracellular calcium increases in hippocampal cells when cultured on coverslips.

Having shown that lymphocytes are not toxic to hippocampal cells, I investigated contact-dependent effects of naïve lymphocytes on modulating neuronal and astrocytic activity. To the best of my knowledge, the effect of naïve lymphocytes on hippocampal activity has not been studied extensively. I have shown for the first time that lymphocytes do not change pharmacological-induced astrocytic $[Ca^{2+}]_i$ increases. However, there was a trend towards impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases following lymphocyte treatment, suggesting a possible effect on glutamate receptors, but this effect was not significant. More experiments should be performed in the future, which would increase n numbers, to support this trend. Having shown that the cytokines IL-16 and IFN-γ could not be detected during lymphocyte treatment, the possible effect on neuronal glutamate receptors observed in this chapter could be mediated via different mechanisms than shown in chapter 4 and thus suggest a possible contact-dependent mechanism. Most contact-dependent interactions between T cells and neurons are cytotoxic and are mediated by either MHC complexes and its subsequent release of perforin and granzymes, or Fas/FasL interactions and LFA-1/ICAM-1 complexes (Siffrin *et al.*, 2010; Zhang *et al.*, 2013; Di Liberto *et al.*, 2018). FasL expression, however, is only upregulated on activated T cells (Brunner *et al.*, 2000) and is therefore considered not involved in contact-dependent effects of naïve lymphocytes on neuronal activity. An immunofluorescent staining for either the MHC class I and II molecules as well as ICAM-1 on neurons could provide more insight into impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases observed following lymphocyte exposure. This would also confirm whether lymphocytes directly target glutamate receptors or whether other signalling pathways are involved. One study has however shown that effector T cells can inhibit LTP (Lewitus *et al.*, 2007), a mechanism mediated by AMPA and NMDA receptors. However, this study confirmed that T cell-mediated inhibition of LTP was mediated by GABA and could be

blocked by the GABA inhibitor picrotoxin. Indeed, T cells are known to synthesize GABA (Bhat *et al.*, 2010) and therefore future experiments should test for the presence of this inhibitory neurotransmitter in co-culture supernatant.

5.3.3 Neuronal synaptic communication is functional between two mouse hippocampal networks.

Given that naïve lymphocytes possibly reduce glutamate-induced n$[Ca^{2+}]_i$ increases via a a possible contact-dependent mechanism, I investigated effects of naïve lymphocytes on synaptic communication, which was tested using microfluidic devices integrated with calcium imaging techniques. To the best of my knowledge, my experiments indicate for the first time that synaptic communication exists between two mouse hippocampal cultures, similar to previously reported in rat hippocampal cultures by our laboratory (Robertson *et al*., 2014, MacKerron *et al*., 2017). Compared to rat hippocampal cultures, mouse neuronal axons, visualised with ß-III-tubulin, start to project their processes into microchannels at 4-5 DIV instead of 3 DIV (Robertson *et al*., 2014). While neurite growth was optimal on PLL-coated coverslips, PLL-coated devices did not provide sufficient support for neurite growth and therefore devices were coated with PLO. In general, neurite growth was similar to other microfluidic experiments used for neuroscience research (Coquinco *et al.*, 2014; Lei *et al.*, 2016; Samson *et al.*, 2016). Furthermore, in agreement with rat cultures used in our laboratory (Robertson *et al*., 2014), synapse formation in mouse hippocampal neurons, visualised by the synaptic vesicle marker synaptophysin, occurred at 12 DIV. Presence of synapses was also similar to other studies using electrophysiological techniques in which synaptic activity is clearly evident at this timepoint. (Gan *et al.*, 2011; Ritchie *et al.*, 2018).

Neuronal synaptic communication between two mouse hippocampal networks was induced via glutamate stimulation. This is in agreement with other studies that have shown pharmacologically-induced synaptic communication between two neuronal populations, albeit

these were reliant on electrophysiological techniques and rat cortical cultures (Kanagasabapathi *et al.*, 2011, Kanagasabapathi *et al.,* 2013). It can however not be ruled out that synaptic communication between two chambers solely occurred via neurites from the direct chamber transferring synaptic activity to neurons in the indirect chamber, as neurites from the indirect chamber are also crossing the microchannel barrier into the direct chamber. Furthermore, glutamate and KCl application in devices have shown that the two mouse neuronal networks are not only connected with each other, but the two neuronal networks are also functionally connected within each compartment, similar to other studies using electrophysiology (Kanagasabapathi *et al*., 2011, Kanagasabapathi *et al*., 2013). My data on intra-compartmentalized connectivity is also in agreement with other studies that have visualized spontaneous activity of human stem cell-derived dopaminergic neurons as well as HIP™ neurons, Dopa.4U™ neurons, iCell® neurons and Axol neurons in microfluidic devices using calcium imaging techniques (Moreno *et al.*, 2015; Wevers *et al.*, 2016).

It should be noted that only two out of six devices derived from 3 hippocampal cultures showed fully functional synaptic communication between both networks. Within the other devices, not all neurons in the indirect chamber had shown increases in $[Ca^{2+}]_i$ levels in response to glutamate application in the direct chamber. Thus, as only a part of the network in the indirect chamber was able to elicit neuronal $[Ca^{2+}]_i$ increases, my data revealed overall no significant synaptic communication in these four devices. This could have been due to improper culture functioning, as I experienced a few problems while setting up mouse hippocampal cultures during later stages of my PhD. Having confirmed that the reagents used to set up hippocampal cultures did not interfere with the functionality of the cultures and that functional cultures could be set up from TLR3 knockout and PLE8 wildtype mice, it was suggested that there were issues with the breeding of C57BL6/J mice. Therefore, BALB/c mice were chosen to perform experiments on synaptic communication using microfluidic devices. Whilst it was decided to use BALB/c instead of C57BL6/J mice, problems with functionality were still

emerging from time to time. However, health of BALB/c hippocampal cultures was in general better compared to the health of C57BL6/J cultures, as calcium imaging of both networks from BALB/c mice showed spontaneous activity during vehicle treatment.

Next to having shown glutamate and KCl-induced neuronal $[Ca^{2+}]_i$ increases in microfluidic devices, I have shown for the first time astrocytic responses to pharmacological stimuli in mouse hippocampal cultures in microfluidic devices. While data from our laboratory has shown $[Ca^{2+}]_i$ increases in astrocytes in response to glutamate (Robertson *et al*., 2014), an astrocyte specific agonist has not been tested yet. Hence, my data reveals both glutamate and ADP-induced $[Ca^{2+}]_i$ increases in astrocytes cultured in microfluidic devices. With the understanding of astrocytic function under both physiological and pathophysiological conditions increasing dramatically (Santello, *et al*., 2012; Verkhratsky *et al.*, 2014), it could be speculated that the methodology used in this chapter could be useful in further increasing our understanding of neuron–astrocyte interactions under these conditions. As such, whilst it is known that astrocytes can release glutamate following astrocytic activity in the form of $[Ca^{2+}]_i$ increases and then modulate neuronal activity (Savtchouk and Volterra, 2018), astrocytic ADP stimulation in my experiments, visualized by increases in astrocytic $[Ca^{2+}]_i$ levels, did not generate neuronal synaptic communication between two networks. There was however, a small percentage of neurons in both the direct and indirect chamber that were active following ADP application, but these corresponding neuronal responses were not strong enough to induce overall synaptic communication between two networks. The lack in synaptic communication following astrocyte specific stimulation is in agreement with another study that has used transgenic mice that express a Gq-coupled receptor only in astrocytes to evoke astrocytic $[Ca^{2+}]_i$ increases using an agonist that does not bind endogenous neuronal receptors in the brain (Fiacco *et al.*, 2007). Stimulating these astrocytes does not increase neuronal $[Ca^{2+}]_i$ levels, produces neuronal slow inward currents, or affects excitatory synaptic activity.

While the system has great potential to show glutamate-induced synaptic communication between two hippocampal networks as has been shown in this chapter, there is a limitation with the protocol used. Typically, calcium imaging experiments are performed on cells coated to coverslips under perfusion with extracellular fluid constantly being refreshed (Bushell *et al.*, 2006; Gan *et al.*, 2011; Rivetti di Val Cervo *et al.*, 2017; Tinning *et al.*, 2017). This perfusion allows for drugs being washed on and off and therefore cell recovery can be monitored, and a second drug can be applied without the previous drug interfering with the second drug. In the experimental protocol I have used, a single application of a stimulus was applied and cells were not allowed to fully recover, thus cells were less likely to respond to a second drug. Indeed, after applying glutamate to neurons in the direct chamber, neuronal $[Ca^{2+}]_i$ levels did not or only slightly increase following application of the second drug KCl. Even though KCl was only applied to discriminate between neurons and astrocytes, a lower or non-response to KCl due to interference of the previous drug made this difficult. Having said that, typically astrocytes do not respond to KCl stimulation as they are not capable of generating action potentials (Wu *et al.*, 2015), as shown in previous experiments using hippocampal cells cultured on coverslips (chapter 4 and 5). Astrocytes cultured in microfluidic devices did respond to KCl application in both the direct and indirect chamber. Elevations in $[Ca^{2+}]_i$ levels in astrocytes can occur spontaneously or following neuronal neurotransmitter release, including glutamate (Perea and Araque, 2005). Hence, many studies have shown that neuronal stimulation triggers $[Ca^{2+}]_i$ oscillations in astrocytes (Pasti *et al.*, 1997). Therefore, it could be that neuronal stimulation with KCl releases the neurotransmitter glutamate that will then stimulate astrocytes, as the neuronal-released glutamate is not washed off and remains within the microfluidic chamber. Having validated a microfluidic perfusion system in our laboratory (MacKerron *et al*., 2017), future experiments should use this platform to confirm results from this chapter.

5.3.4 HCM and lymphocyte exposure do not support synaptic communication.

Given the trend towards lymphocyte-induced impaired glutamate-induced $[Ca^{2+}]_i$ increases in neurons cultured on coverslips but no effect on glutamate- or ADP-stimulated astrocytes, I went on to confirm this in microfluidic devices. I have shown that glutamate-induced $[Ca^{2+}]_i$ increases in neurons is not reduced following lymphocyte exposure in microfluidic devices. In agreement with absent effects of lymphocytes on astrocytes cultured on coverslips, lymphocyte treatment in microfluidic devices was without effect on glutamate- and ADP-induced astrocytic $[Ca^{2+}]_i$ increases. A possible explanation for the observed differences between lymphocyte exposure of hippocampal cells cultured on coverslips and within microfluidic devices could be differences in mouse strain. Whilst *in vitro* and *in vivo* calcium imaging of BALB/c mice has been shown successful (Stosiek *et al.*, 2003), significant differences have been observed between C57BL6/J and BALB/c mice, particularly in activity amplitude and correlation between activities in different hippocampal subregions (Jansen *et al.*, 2009). Thus, more experiments comparing both mouse strains plus both techniques should be performed in the future to fully confirm observed effects. While functional synaptic communication was evident between two mouse hippocampal culture networks, both HCM control as well as lymphocyte exposure of hippocampal cells cultured in microfluidic devices did not support glutamate-induced synaptic communication. Therefore, it would be good to test the effects of HCM control exposure on fully active cultures from newly bought-in mice.

5.3.5 CCL19 does not support lymphocyte migration in microfluidic devices.

To study lymphocyte migration following increased neuronal activity or cell death in order to better understand lymphocyte migratory behaviour in the CNS under pathological conditions, I first examined whether it was possible to study lymphocyte migration *per se* in microfluidic devices. My data shows that lymphocytes do not adhere as well to PLO-coated devices as to collagen-coated devices. PLO-coated coverglass has been used to measure changes in $[Ca^{2+}]_i$ levels in lymphocyte cultures (De Rosa *et al.*, 2009), but whether PLO coating supports

lymphocyte migration is unknown. Lymphocyte adherence can be ensured using fetal bovine serum in culture medium as it includes all factors needed for cell attachment (Brunner *et al*., 2010). However, it is crucial to avoid the use of serum for culturing synaptically active hippocampal cells (Kim *et al.*, 2013). As it was intended to test lymphocyte migration following increased neuronal activity or neuronal cell death, serum-free medium was required to address this first question.

As it is known that CCL19, a ligand of the receptor CCR7 on naïve and activated immune cells, induces chemokinesis *in vitro* in both transwell assays (Okada and Cyster, 2007) and in microfluidic devices (Schwarz *et al.*, 2016), I tested CCL19-induced lymphocyte migration in microfluidic devices without the presence of hippocampal cultures as a proof of principle. Lymphocyte migration towards a CCL19-containing chamber however was not observed up to 24 hours *in vitro*. Although, one device showed lymphocytes gathering near microchannels following 3 hours in vitro. It was shown that this was due to device tilting, which creates a small fluid flow towards the opposite chamber, pushing cells towards the microchannels, instead of actual cell migration towards CCL19. To better understand why lymphocytes would not migrate towards the CCL19-containing chamber, CCR7 expression on lymphocytes would need to be confirmed. Furthermore, it could be that the gradient is too strong and therefore CCL19 will disperse quickly into the other chamber and lymphocytes will therefore not migrate. To confirm that a stable concentration gradient was established with CCL19, FITC-dextran can be used as a proxy to monitor chemokine gradients within the chamber, as this is suggested to have a similar diffusion profile (Schwarz *et al*., 2016). However, all this was not possible due to time limitations.

In conclusion, it cannot be established from results presented in this chapter whether lymphocytes modulate neuronal and astrocytic function, or neuronal synaptic communication in a contact-dependent mechanism. However, presented data does show that naïve lymphocytes are not toxic to hippocampal cells.

Chapter 6

General discussion.

6.1 Major findings.

The burden of CNS disorders has increased substantially over the last decades, including a 36.7% increase in the number of deaths between 1990 and 2015 and is expected to keep rising due to a prolonged life expectancy (GBD Group, 2017)). Hence, CNS disorders are the second-leading cause of deaths in 2015 and account for 10.2% of global disability-adjusted life-years (DALYs), a measure of overall disease burden expressed as the numbers of years lost due to ill-health, disability or early death (GBD Group, 2017). As the CNS is extremely complex and most CNS disorders have a multifactorial aetiology, development of effective pharmacological therapies remains extremely challenging. Recently, there has been intense interest in the role of inflammation in CNS disorders, not only those that are regarded as autoimmune diseases, including MS, but also in neurodegenerative diseases, including AD and stroke. Whilst our understanding of the diverse role of inflammatory processes in CNS disorders is advancing, there still remains a void in how to target these inflammatory processes to arrest disease progression, promote CNS parenchymal cell repair or even prevent disease onset. Therefore, objectives for clinical intervention of CNS disorders are to broaden our understanding of the interplay between neurons and lymphocytes. In this thesis, my main aim was to investigate whether naïve lymphocytes affect neuronal activity in a contact-dependent or -independent manner. In addition, I examined whether alterations in neuronal activity or neuronal apoptosis/necrosis influences lymphocyte migration. In order to address these fundamental aims, a viable experimental set up required for *in vitro* co-culture experiments was developed. Major findings in this thesis are listed below (Figure 6.1).

Chapter 3.

- Lymphocytes survive in cNeurobasal-A medium.
- Lymphocyte cell types present in lymph node cell preparations cultured in cNeurobasal-A medium are similar to those cultured in cRPMI medium.
- $CD4^+$ Th cell proliferation in cNeurobasal-A medium is identical in cRPMI medium.

- Lymphocyte-derived cytokine release in cNeurobasal-A medium is comparable in cRPMI medium.
- Constitutive IL-16 release in lymph node cell preparations is not confined to one cell type.

Chapter 4.

- LCM impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases.
- HLCM does not change pharmacological-induced neuronal or astrocytic $[Ca^{2+}]_i$ increases.
- IL-16 present in LCM and HLCM does not alter basal neuronal or astrocytic $[Ca^{2+}]_i$ levels.
- IL-16 treatment shows a trend towards impaired glutamate- but not KCl-induced neuronal $[Ca^{2+}]_i$ increases.
- LCM-induced impaired neuronal intracellular calcium increases are CD4-independent.

Chapter 5.

- Lymphocyte treatment shows a trend towards impaired glutamate-induced neuronal but not astrocytic $[Ca^{2+}]_i$ increases in a contact-dependent manner.
- Glutamate application induces neuronal synaptic communication between two environmentally isolated networks in microfluidic devices.
- HCM and lymphocyte treatment does not support glutamate-induced neuronal synaptic communication.
- Lymphocytes do not induce hippocampal cell death.

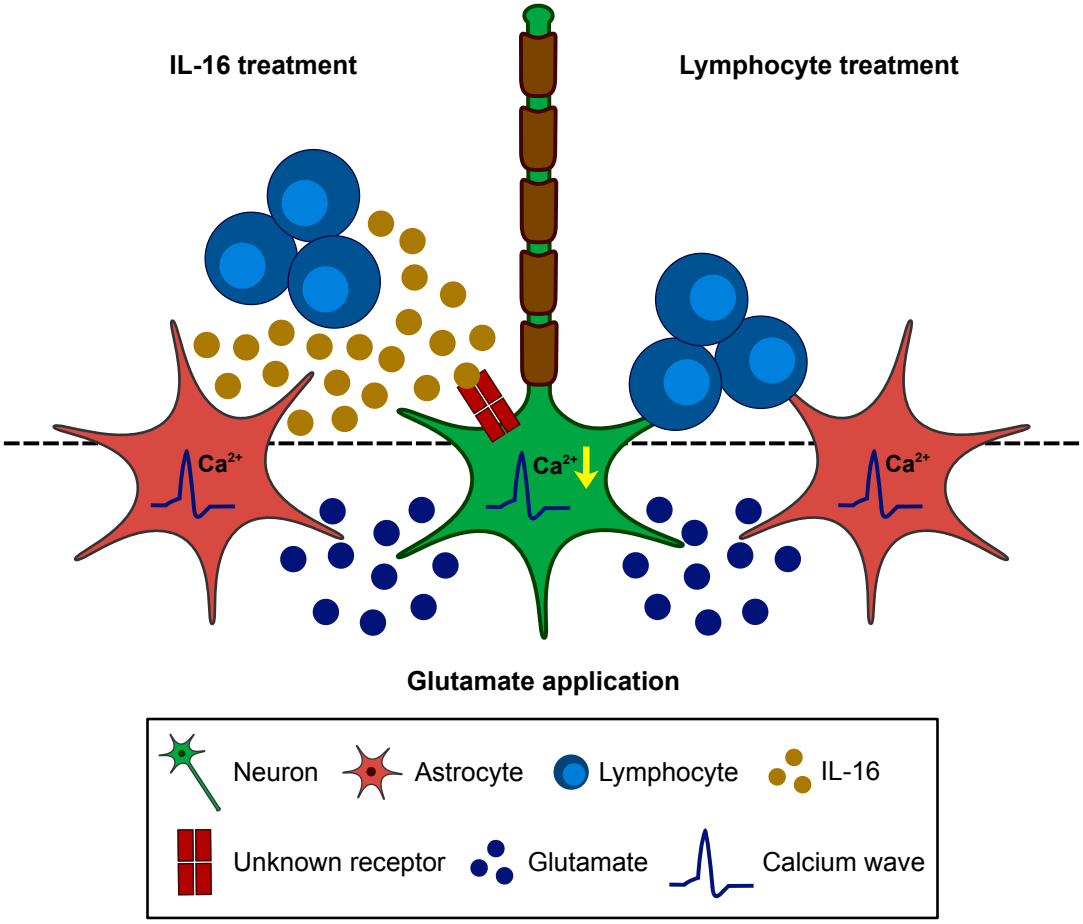

Figure 6.1. Presented data in this thesis show a trend towards impaired glutamate-induced neuronal but not astrocytic $[Ca^{2+}]_i$ increases following IL-16 and lymphocyte treatment.

6.1.1 Neuronal-lymphocyte co-culture experiments can be conducted in cNeurobasal-A medium to study contact- and IL-16-dependent lymphocyte effects on hippocampal activity.

Studying cell-cell interactions calls for a tight control of the environment in *in vitro* experiments, especially with co-cultures of different cell types that require distinct growth media. Considering that cultured neurons are highly susceptible to changes in their environment, initial experiments were performed to confirm that lymphocyte viability and functionality does not change in serum-free Neurobasal-A medium containing B27 supplement and L-glutamine, conventionally used for post-natal and adult brain neurons. Here, I have shown that cNeurobasal-A medium maintains lymphocyte viability and that cell types

present in lymph node cell preparations cultured in cNeurobasal-A medium are similar to those cultured in conventionally used cRPMI medium. Furthermore, lymphocytes cultured in cNeurobasal-A medium were able to proliferate and secrete cytokines comparable to those cultured in cRPMI medium. This suggests that co-culture experiments with hippocampal cultures and naïve lymphocytes can be conducted in cNeurobasal-A medium. Most studies investigating neuron-T cell interactions do not address experimental medium optimisation and it is not clear from their methods section which medium is used for co-incubation experiments, as immune cells were cultured in a different medium than neuronal cultures (Medana and Gallimore 2000, Medana *et al*., 2001, Giuliani *et al.*, 2003, Shrestha *et al*., 2006, Meuth *et al*., 2009, Chevalier *et al*., 2011). However, there is one study that clearly stated that $CD4^+$ T cells were added in RPMI-growth medium to cortical neurons for 24 hours (Ishii *et al*., 2010) but did not specify whether RPMI medium had any negative effects on the health of the cultures. It was also not clear form this study whether $CD4^+$ T cells in RPMI where added to neurons in Neurobasal medium or that Neurobasal medium was replaced with $CD4^+$ T cells in RPMI. Furthermore, two other studies have added T cells in artificial CSF (ACSF) to acute hippocampal slice cultures in ACSF (Nitsch *et al*., 2004, Lewitus *et al*., 2007). However, as our laboratory has discovered that T cells are not motile in artificial HBBS based solutions, which was a requirement for my lymphocyte migration studies, this was not an option. Thus, my data shows for the first time a detailed analysis of the ideal experimental conditions for lymphocyte-neuron co-culture experiments.

6.1.2 Lymphocyte-derived IL-16 possibly reduces neuronal activity in a CD4-independent manner.

A novel finding in my thesis is that LCM impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases. To the best of my knowledge the use of LCM to better understand contact-independent lymphocyte actions on neuronal and astrocytic functions has not been extensively investigated. There is one study that showed that culture supernatant from lymphokine-

activated killer (LAK)-T cells causes neurogenesis when administered to adult rats (Ishiguro *et al.*, 2016). Whether my data, showing that LCM induces glutamate receptor modulation, corresponds with neurogenesis is not clear, as the role of glutamate and glutamate receptors in neurogenesis is ambiguous since glutamate activates neurogenesis and glutamate receptor stimulation inhibits neurogenesis (Nacher and McEwen, 2006). It is however unlikely, as the same study showed that $[Ca^{2+}]_i$ levels were increased following LAK-T supernatant administration to hippocampal slice cultures (Ishiguro *et al*., 2016), which is in contrast to my data. A different study showed that conditioned medium from concanavalin A-activated lymphocytes maintains sympathetic neuronal survival in culture (Gozes *et al.*, 1982). Together with the results previously found in our laboratory showing that LCM is neuroprotective of KA- and OGD-induced cell death (Shrestha *et al*., 2014), it could be suggested from my data that lymphocytes are neuroprotective by dampening the calcium influx via glutamate receptor modulation in a contact-independent manner. This contact-independent manner was further investigated by quantifying the concentration of IL-16 in LCM, as this was the only cytokine upregulated in LCM-mediated neuroprotection (Shrestha *et al*., 2014). Indeed, IL-16 was quantified at 300 pg mL^{-1} in LCM and it was shown that IL-16 was not taken up by hippocampal cells during LCM treatment. Whilst IL-16 induces increases in $[Ca^{2+}]_i$ levels in T cells, my data reveals no effect of rIL-16 (300 pg mL^{-1}) on neuronal or astrocytic basal $[Ca^{2+}]_i$ levels. Exposure of hippocampal cultures to rIL-16 for 1 hour results in reduced glutamate-induced neuronal $[Ca^{2+}]_i$ increases but was not significant. KCl-induced neuronal $[Ca^{2+}]_i$ increases were not affected by rIL-16 exposure and therefore it is suggested that IL-16 might underlie the effects observed with LCM exposure. Together with additional data found in our laboratory, showing that IL-16 reduces phosphorylation of GluA1 at the S831 site (Hridi *et al*., 2019) and given that KA is an agonist for both KA and AMPA receptors (Wang *et al.*, 2005), my data might indicate that IL-16 is neuroprotective of KA-induced neurotoxicity. Whether IL-16 directly targets glutamate receptors or this is mediated via a different receptor still needs to be further investigated. I have however shown that IL-16-induced impaired

neuronal $[Ca^{2+}]_i$ increases is not dependent on CD4 or CD9 receptors (Hridi et al., 2019). This is in agreement with other studies that have shown that IL-16 does not solely interact with the CD4 receptor. As such, IL-16 induces cytokine production and migration in $CD4^{-/-}$ T cells (Mathy *et al.*, 2000) as well as neuronal outgrowth in $CD4^{-/-}$ CGNs (Fenster *et al.*, 2010). Thus, my data revealing that lymphocytes interact with neurons in a contact-independent manner possibly by IL-16, which then impairs glutamate-induced neuronal $[Ca^{2+}]_i$ increases via CD4-independent mechanisms further supports this previously mentioned perspective. Whilst the exact mechanism is not yet fully understood, IL-16 offers promising opportunities to be developed into an effective drug that arrests glutamate-toxicity found in many neurodegenerative diseases including AD, ALS and Huntington's disease (Lewerenz and Maher, 2015). However, the fact that IL-16 is detected at sites of Th1-mediated inflammation in MS, I/R injury and tissue transplant rejection and has been known to induce inflammation and disease progression (Hall *et al.*, 2016) implicates that IL-16 can play a biphasic role. Therefore, therapeutic efficiency of IL-16 needs to be carefully investigated in different disease models.

6.1.3 Lymphocytes possibly impair neuronal activity in a contact-dependent manner, without causing lymphocyte-induced cell death.

Whilst most studies focus on effector or memory T cells in CNS homeostasis or disorders, I present novel data revealing that naïve lymphocyte treatment shows a trend towards impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases, however it has to be mentioned that presented results were not significantly different. As the cytokines IFN-γ as well as IL-16 are below detectable levels during lymphocyte exposure of hippocampal cultures, my data suggests that the possible lymphocyte-mediated impaired effects on glutamate-induced neuronal $[Ca^{2+}]_i$ increases are contact-dependent. Whilst it is shown that activated lymphocytes either induce neuronal cell death, enhance neurite outgrowth, inhibit pathology or clear viruses from neurons

in various contact-dependent manners (Giuliani *et al*., 2003, Meuth *et al*., 2009, Suzuki *et al*., 2010, Ishii *et al*., 2010, Shrestha *et al*., 2012, Zhang *et al*., 2013), the interplay between naïve lymphocytes and neurons has not been investigated extensively. This could be due to the fact that naïve lymphocytes found in the CNS via the BCSFB are considered to be part of the recirculating lymphocyte pool, which are immediately drained from the CNS into cervical lymph nodes once they do not encounter their corresponding antigen (Seabrook, *et al*., 1998). Several studies have shown though that naïve T cells do not induce neurotoxicity, while activated lymphocytes did in a contact-dependent manner in these studies (Giuliani *et al*., 2003, Nitsch *et al*., 2004, Haile *et al*., 2011, Haile *et al*., 2015). Furthermore, naïve T cells were not able to enhance neurite outgrowth, which was achieved when cortical neurons were co-cultured with activated lymphocytes (Ishii *et al*., 2010). Thus, it is not clear whether naïve T cells are neuroprotective or just cause no harm to neurons, as lymphocytes did not induce hippocampal cytotoxicity. The exact contact-dependent mechanisms involved still need to be further investigated.

My data also indicates that neuronal synaptic communication between two mouse hippocampal networks in microfluidic devices can be induced via glutamate stimulation. This is the first time that functional synaptic communication in an *in vitro* mouse model was monitored by integrating microfluidic devices with calcium imaging techniques. Our laboratory has mainly shown functional synaptic communication between rat hippocampal culture networks (Robertson *et al*., 2014, Samson *et al*., 2016, MacKerron *et al*., 2017). Similar pharmacologically-induced synaptic communication was achieved between two rat cortical neuronal networks measured by electrophysiological techniques integrated in microfluidic devices (Kanagasabapathi *et al*., 2011, Kanagasabapathi *et al*., 2013). Other studies have shown the use of integrating microfluidic devices with calcium imaging techniques to study neuronal activity, however neurons were only cultured in one chamber, therefore synaptic communication between different networks was not investigated in these microfluidic platforms (Moreno *et al.*, 2015; Wevers *et al.*, 2016). Interestingly, data gathered in this thesis

thus allows for the investigation of how the activity of one hippocampal cultured network is modulated by changes in activity in a second, environmentally isolated, network via functional synaptic connections. Hence, I exposed one network to naïve lymphocytes and measured neuronal and astrocytic activity in both networks via calcium imaging techniques. Although data presented in this thesis could not confirm effects of lymphocytes on glutamate-induced neuronal $[Ca^{2+}]_i$ increases, contact-dependent interactions between lymphocytes and hippocampal cells do not mediate lymphocyte-induced cell death. This confirms the suggestion that naïve T cells do not induce neuronal excitotoxicity in contrast to activated T cells (Giuliani *et al*., 2003, Nitsch *et al*., 2004, Haile *et al*., 2011, Haile *et al*., 2015).

6.1.4 Lymphocyte migration following changes in neuronal activity or neuronal apoptosis.

Recently, immune cell migration towards tumour cells via the release of soluble mediators has been studied using microfluidic devices (Mattei *et al.*, 2014). Interestingly, splenocytes were cultured separately from melanoma cells using three separate chambers. These chambers were fluidically connected via microchannels and allowed for the distribution of soluble mediators between chambers. Furthermore, it has been shown that activated human blood T cells migrate towards the cathode of direct current electric fields in microfluidic devices (Li *et al.*, 2011). With this in mind, I aimed to translate this to a neuronal platform and study lymphocyte migratory behaviour following increased neuronal activity or cell death. I first had to examine whether it was possible to study lymphocyte migration *per se* in microfluidic devices. Therefore, I tested whether naïve lymphocytes would migrate towards the chemoattractant CCL19. Whilst it is shown in both transwell assays and microfluidic devices that CCL19 induced chemokinesis of naïve as well as activated lymphocytes containing the CCR7 receptor (Okada and Cyster, 2007; Schwarz *et al.*, 2016), my data did not confirm these results in the microfluidic platform I used. This platform is different from the platform used by Schwarz and his colleagues, however we both used static gradients by using a switching source-sink flow

pattern (Frank and Tay, 2013; Kramer *et al.*, 2013). To confirm that a stable concentration gradient was established with CCL19, FITC-dextran can be used as a proxy to monitor the chemokine gradient within the chamber, as this is suggested to have a similar diffusion profile (Schwarz *et al*., 2016). In addition, the use of different extracellular matrix complexes should be tested to optimise lymphocyte migration. Once lymphocytes effectively migrate towards the well-known chemoattractant CCL19, more complex environments can be tested to address the fundamental questions regarding neuronal activity- or cell death-induced lymphocyte migration in the brain. As such, *in vitro* BBB structures on a chip is a recently developed method to mimic brains *in vivo* that simulates brain activities and CNS environment. This *in vitro* brain model consists of two membrane-free compartments, including an endothelial cell-lined vascular compartment and an ECM-embedded brain tissue compartment comprised of neurons, astrocytes and microglia (Brown *et al.*, 2015; Koo, *et al.*, 2018). It would be interesting to add lymphocytes to the endothelial vascular compartment in order to create ideal conditions for lymphocytes to arrest motility, adhere to endothelial cells and be able to migrate towards the brain tissue compartment. Whether lymphocyte migration is possible in this platform, chemoattractant concentration gradient experiments (Nandagopal, Wu and Lin, 2011) should be tested before changes in neuronal activity or induction of cell death could be investigated to induce lymphocyte migration.

6.2 Limitations of the presented study.

Although data presented in this thesis advances our understanding of the interactions between lymphocytes and hippocampal cells, a number of limitations and technical considerations exist and need to be considered for future studies. First, as the focus of this thesis is on neurodegeneration and involvement of inflammation in associated diseases, a phenomenon that occurs mostly later in life and can arise from age-dependent pathologies, the use of neonates is not optimal to study the interaction between the immune system and the CNS. However, successful culturing of neuronal cultures from adult animals is still difficult and

therefore it was decided to use postnatal mouse primary hippocampal neuronal cultures in this study, which is widely accepted as a methodology to study neurological aspects (Kaar, Morley and Rae, 2017). Another weakness of the work presented here is the low sample size numbers (n=3 independent cultures). This should be increased as nowadays issues of data reliability arise using fewer than n=5/group (Curtis *et al.*, 2015). Furthermore, quantified analysis should have been performed on data presented in Figure 4.1 to reliably conclude that the neuron/astrocyte ratio in mouse hippocampal cultures used in this thesis is similar to the ratio used in other studies in our lab using rat cultures. Last, as this thesis is in part a follow up on the work performed by Shrestha et al., 2014, an experiment should have been included that investigated the effects of rIL-16 on kainate-induced neurotoxicity, especially as the results presented in this thesis suggest a neuroprotective role for IL-16. Although this would have been an interesting discovery, time wise it was not possible to perform this experiment and would need to be addressed in future experiments.

6.3 Future studies.

To further investigate the exact mechanisms by which IL-16 preserves neuronal activity and the contact-dependent interaction between naïve lymphocytes and hippocampal cells, unresolved questions need to be answered, which are listed below.

- Is IL-16 the only mediator of impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases? This could be tested by blocking experiments, including the application of rIL16 in the presence and absence of an anti-IL-16 antibody.
- Do different IL-16 concentrations modulate neuronal and astrocytic $[Ca^{2+}]_i$ levels? If so, what is the optimal concentration? This could be investigated by testing the concentrations used that revealed IL-16-induced increases in $[Ca^{2+}]_i$ in T cells (Cruikshank et al., 1991) or test the quantified concentration used in this thesis on $[Ca^{2+}]_i$ levels in T cells.

- Does IL-16 directly target AMPARs? This could be tested by applying rIL-16 in the absence and presence of AMPAR antagonists to hippocampal cultures for 1 hour prior to calcium imaging experiments.
- Does PKC translocation underlie the IL-16-mediated impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases? This could be examined by applying rIL-16 in the absence and presence of PKC inhibitors to hippocampal cultures for 1 hour prior to calcium imaging experiments.
- Which receptor is involved in IL-16-mediated impaired glutamate-induced neuronal $[Ca^{2+}]_i$ increases? This could be investigated by confirming that IL-16 effect are CD4-independent by using CD4-knockout mice or applying rIL-16 in the absence and presence of CD4 blocking antibodies to hippocampal cultures for 1 hour prior to calcium imaging experiments.
- Are the contact-dependent naïve lymphocyte-neuronal cell interactions mediated via MHC/antigen-dependent or -independent mechanisms? This could be investigated by staining for MHC I or II molecules, Fas or ICAM-1 in hippocampal cultures or perform a western blot to determine protein expression of these molecules. Furthermore, lymphocytes can be applied in the presence and absence of MHC I, MHC II, Fas and ICAM-1 blocking antibodies to hippocampal cultures for 1 hour prior to calcium imaging experiments.
- Do naïve lymphocytes directly target GluRs and which GluR is involved? This could be tested by applying lymphocytes in the absence and presence of AMPAR, KAR and NMDAR antagonists to hippocampal cultures for 1 hour prior to calcium imaging experiments.
- Is there a role for GABA and its receptor in the possible lymphocyte-mediated reduction in glutamate-induced neuronal $[Ca^{2+}]_i$ increases? This could be examined by performing an ELISA to measure GABA levels in the supernatant from lymphocyte-neuronal co-

cultures. Furthermore, western blots using anti-GAT antibodies can be performed to address the questions regarding endocytosis of GATs on astrocytes.

- Are astrocytes involved in the observed lymphocyte-mediated reduction in glutamate-induced neuronal $[Ca^{2+}]_i$ increases? This could be investigated by blocking astrocyte activity using the glial toxin FAc before hippocampal cultures are exposed to lymphocytes and calcium imaging is performed.

Publications.

Papers.

Hridi, S. U.*, **Franssen, A. J. P. M.***, Jiang, H. R. & Bushell, T. J. Interleukin-16 inhibits sodium channel function and GluA1 phosphorylation via CD4-independent mechanisms to reduce hippocampal neuronal excitability and synaptic activity. *Mol. Cell. Neurosci.* 2019, pp.71-78 *shared first author.

Schniete, J., **Franssen, A.**, Dempster, J., Bushell, T., Amos, W. & McConnell, G. Fast optical sectioning for widefield fluorescence mesoscopy with the mesolens based on HiLo microscopy. *Sci. Rep.* 2018, pp. 16259.

Tinning, P. W., **Franssen, A. J. P. M.**, Hridi, S. U., Bushell, T. J. & McConnell, G. A 340/380 nm light-emitting diode illuminator for Fura-2 AM ratiometric Ca^{2+} imaging of live cells with better than 5 nM precision, *J. Microsc,* 2017, 269(3), pp. 212–220.

Poster presentation.

Franssen, A. J. P. M., Hridi, S. U., Millington, O. R., Jiang, H. R. & Bushell, T. J. Lymphocytes modulate hippocampal neuronal but not astrocytic activity in a contact-dependent manner and via soluble mediators. Poster B.022, Federation of European Neuroscience Societies (FENS) Forum 2018, Berlin (Germany).

Franssen, A. J. P. M., Millington, O. R., Jiang, H. R. & Bushell, T. J. IL-16 modulates hippocampal neuronal and astrocytic activity via a CD4-independent mechanism. Poster 317, British Society for Immunology (BSI) Congress 2017, Brighton (United Kingdom).

Oral presentation.

Franssen, A. J. P. M.*, Hridi, S. U.*, Jiang, H. R., Bushell, T. J. Interleukin-16 inhibits sodium channel function and GluA1 phosphorylation via CD4-independent mechanisms to reduce hippocampal neuronal excitability and synaptic activity. *Scottish Neuroscience Group (SNG) Meeting* 2018, Dundee (United Kingdom).

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