INVESTIGATING THE ROLE OF LYMPHOCYTES IN *IN VITRO* MODELS OF NEURODEGENERATIVE DISEASE

RAJEEV SHRESTHA

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Abstract

The CNS was until recently considered an 'immune-privileged' area. However, evidence now exists that inflammation and immune cells play key roles in several inflammation-related CNS disorders. Despite this, the exact role of immune cells under physiological and pathophysiological conditions remains elusive. Hence, I sought to investigate the role of lymphocytes using *in vitro* models of neurodegenerative disease in organotypic slice cultures.

Organotypic cortico-hippocampal slice cultures (OSCs) were prepared from mice (4-7 days old) and maintained for 13-15 days in vitro. Neuronal death was assessed utilising propidium iodide (PI) fluorescence following either an excitatory insult (300μM Kainate (KA), 1hr) or oxygen-glucose deprivation (OGD; 30 mins) in the absence and presence of different lymphocyte preparations. Western-blotting and a pharmacological approach were implemented to investigate possible signalling pathways.

Lymphocytes significantly reduced KA-induced toxicity and OGD-induced cell death under the present experimental conditions indicating a neuroprotective role for lymphocytes. In addition, conditioned media from lymphocytes were also neuroprotective against KA-induced toxicity and OGD-induced cell death. This finding suggests that lymphocyte-mediated neuroprotection is contact-independent. Furthermore, pre-treatment of OSCs with FAc, a glial cell metabolic inhibitor, abolished the observed neuroprotection in the KA model but not in the OGD model. In addition, western blotting revealed that in slices co-cultured with lymphocytes there is a significant reduction in the activity of ERK and p38 MAP kinase.

The present data indicates that in two different models of neurodegenerative disease, lymphocytes are neuroprotective under my experimental conditions, an effect mediated, at least in part, by activation of astrocytes and an inhibition of MAP kinase signalling pathways. However, further work is needed to establish the involvement of microglia and other immune cells including macrophages and natural killer cells.

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Author's declaration

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List of Abbreviations

µl	micro liter
µM	micro molar
µm	micrometer
^{0}C	degree Celsius
AD	Alzheimer's disease
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APCs	antigen presenting cells
APs	action potentials
APS	ammonium persulphate
ATP	adenosine triphosphate
Aβ	amyloid-β
BBB	blood brain barrier
BCR	B cell receptor
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CAM	cell adhesion molecules
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CNP	2', 3'-cyclo nucleotide 3'-phosphodiasterase
CNS	central nervous system
CSF	cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DIV	day *in vitro*
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ECL	enhanced chemiluminescence
EDTA	ethylene-di-amine-tetra-acetic acid

ERK	extracellular signal regulated kinase
FAc	sodium fluoroacetate
FACS	fluorescence-aided cell sorting
FCS	foetal calf serum
GFP	green fluorescent protein
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal axis
hr	hour
HS	horse serum
HSP	heat shock protein
ICAM	intercellular cell adhesion molecule
ICOS	inducible T cell co-stimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IS	immunological synapse
ITAM	immunoreceptor tyrosin-based activation motif
JAM	junctional adhesion molecule
JNK	c-Jun N-terminal kinase
KA	Kainic acid or kainate
LFA	lymphocyte-associated function antigen
LPS	lipopolysaccharides
LT	lymphotoxin
MACS	magnetic assorted cell sorting
MAG	myelin associated protein
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
mg	milligram

MHC	major histocompatibility complex
ml	milliliter
mM	millimolar
MMP	matrix metalloproteinase
MOG	myelin-oligodendrocyte protein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropuridine
MS	Multiple Sclerosis
NF	neurotrophic factor
NK	natural killer
nm	nanometer
NO	nitric oxide
NT	neurotrophin
OGD	oxygen-glucose deprivation
OMgP	oligodendrocyte myelin protein
OSC	organotypic cortico-hippocampal slice culture
OVA	ovalbumin
PBS	phosphate buffer solution
PD	Parkinson's disease
PFA	paraformaldehyde
PG	proteoglycan
PI	propidium iodide
PLP	proteolipid protein
PMSF	phenylmethylsulfonyl fluoride
PSGL	P-selectin glycoprotein ligand
RAG	recombinase activating gene
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulphate
SEM	standard error of mean
TCR	T cell receptor
TEMED	tetramethylethylenediamine

TGF	transforming growth factor
Th	T helper
TIMP	tissue inhibitors of metalloproteinase
TNF	tissue necrosis factor
TNFR	tissue necrosis factor receptor
Treg	regulatory T cell
VCAM	vascular cell adhesion molecule
ver	version

1 Introduction

My research crosses two major areas of neuroscience and immunology for which I have investigated the role of lymphocytes in neurodegenerative diseases. In this chapter, first, I have explained the brief introduction of central nervous system (CNS) and immunology. Following the brief introductions, I have given evidence for the bi-directional communication between the CNS and the immune system. This chapter further describes the role of lymphocytes in normal and diseased state with respect to previous studies. The last part of this chapter has included the scope and importance of this study proposing its hypothesis with the specific aims to address the hypothesis.

1.1 Basic central nervous system

The CNS is considered as a complex organ system consisting of billions of neurons having points of connections more than their number. The immense capacity and ability to remodel itself in response to experience and the environment always fascinate neuroscientists, who believe that this organ system is the most complicated machine ever known to the universe. The ability to receive, retain, recall and analyze complex information is a wonderful capacity of the nervous system (Weil *et al.*, 2008).

The CNS has bi-symmetrically similar structures. The CNS is divided into seven parts; spinal cord, medulla oblongata, pons, cerebellum, mid-brain, diencephalon and the cerebral hemisphere (Kandel, 2000; Bear *et al.*, 2001). It is now well accepted that these different regions have their own specialized functions in the CNS. The spinal cord is the most caudal part of the CNS which receives and processes sensory information from peripheral organs such as skin, muscles and joints and transmits motor commands to those organs. It is further sub-divided into cervical, thoracic, lumbar and sacral regions. The brain-stem precedes the spinal cord and plays a major role in communication between brain and spinal cord. Brain-stem comprises of three structures; medulla oblongata, pons and mid-brain. The medulla oblongata lies just above the spinal cord

and regulate breathing and blood pressure. It is also responsible for taste, hearing and balancing the body. The pons lies superior to medulla oblongata and ventral to the cerebellum. It transfers the information regarding movement and sensation from cerebral cortex to the cerebellum. It is also involved in respiration, sleep and taste. The midbrain lies above the pons and has an important role in regulating voluntary movements as well as sensory and motor functions such as eye movement, visual and auditory reflexes. The cerebellum is situated behind the pons and has several connecting points with the brain stem. It contributes to the control and coordination of motor function (Holmes, 1939) along with motor learning (Thompson and Kim, 1996; Thach, 1998) and cognitive function (Leiner *et al.*, 1993; Rondi-Reig *et al.*, 2005). The diencephalon lies above the midbrain and plays an important role in information processing and regulating autonomic, endocrine and visceral functions. It also regulates circadian rhythms of an individual. The cerebral hemispheres are the largest structure in the brain and consists of outer cerebral cortex and deep lying three structures; the basal ganglia, the amygdala and the hippocampus. These structures are mainly responsible for motor and cognitive functions including memory and emotion. The basal ganglia are responsible for controlling of fine movements and the amygdala is related to social behaviour along with emotions while the hippocampus is concerned with memory. The outer cerebral cortex is also referred to as the neocortex and divided into the frontal lobe, the parietal lobe, the temporal lobe, the occipital lobe and the insular lobe all which possess distinct functions. The frontal lobe deals with planning future action and controlling movement; the parietal lobe is responsible for orientation of objects and various sensations including visual, vestibular, auditory and somatic; the temporal lobe is involved in auditory perception and processing of semantics in speech and vision; the significant function of the occipital lobe involves vision as the primary visual cortex is situated in this region; the insular cortex is responsible for interoceptive awareness and regulation of homeostasis including the immune system (Felleman and Van Essen, 1991; Amaral, 2000; Kandel, 2000; Critchley *et al.*, 2004; Zilles *et al.*, 2004; Pacheco-Lo´pez *et al.*, 2005).

Therefore, it is evident that many sensory, motor and cognitive functions are controlled by more than one region of the brain. This signalling process of the CNS is accomplished by the nerve cells and their connecting points to each other. Morphologically, these nerve cells have a very simple structure and are the basic units of the CNS (Bullock, 1959; Fodstad, 2002). There are different types of nerve cells, which share the same basic architecture. In the nervous system, there are mainly two types of cellular structures and these are neurons and glial cells.

1.1.1 The Neuron

Neurons are also known as nerve cells and it is estimated that 10^{11} neurons are present in the human brain (Kandel, 2000). They are the basic signalling unit of the nervous system (Bullock, 1959; Brodal, 1981) and are the only unit in the nervous system that can transmit or conduct nerve impulses (Fodstad, 2002). The neuron has four distinctive and defined regions which comprise of the cell body, dendrites, the axon and axon (pre-synaptic) terminals (Figure 1.1). These different regions play a major role during signalling processing. The cell body is more prominent with distinct structures of rough endoplasmic reticulum and Golgi complex. Most proteins are synthesised in the cell body which are then transported from neuronal cytoplasm to dendrites and axons via microtubules (Brady, 1991; Cleveland and Hoffman, 1991; Guzik and Goldstein, 2004). These microtubules run along the length of axons and dendrites. On the basis of their shape, neurons are named as unipolar, bipolar or multi-polar neurons. The unipolar neurons are predominant in invertebrates while mutipolar neurons are predominant in vertebrates (Kandel, 2000).

Two kinds of processes arise from the neuronal cell body and these are axons and dendrites. Usually, a neuron consists of one axon which is tubular in shape and extends to form a synaptic connection with other neurons. It divides into a number of branches before making a synaptic connection and transfers signals to the neighbouring neurons via the axon. The point where two neurons communicate is known as a synapse. The

neuron transmits the signal through axon terminals or pre-synaptic terminals to other neurons that receive the signal via dendrites. The pre-synaptic terminals do not have direct contact with post-synaptic soma or dendrites but these terminals are separated with small spaces known as the synaptic cleft (Bullock, 1959; López-Muñoz *et al.*, 2006). This historic finding is mentioned in the first issue of *Revista Trimestral de Histolog ́ıa Normal y Patol ́ogica, May 1888*. The axon terminal ends either at the neuron's dendrites or the cell body or even at the axon of a receiving cell. Neurons are divided into two groups according to their types of axon: axons of one type are wrapped with myelin sheath and others do not have a myelin sheath. The myelin sheath wrapped those neurons at the intermittent intervals and the unwrapped axolemma is referred as 'node of Ranvier' (Girault and Peles, 2002). This architecture enhances the speed of transmission of signals from one neuron to the other neurons. This process of signalling transmission is known as saltatory movement and signals are called action potentials (APs). In saltatory conduction, APs jump from one node to another preventing whole axon depolarisation and lead to fast conduction of signals to target neurons and was first described more than the half century ago (Huxley and Stãmpfli, 1949).

Dendrites are fine processes which spread to target neurons. This enables neurons to sense the changes in the microenvironment of other neurons (Kandel, 2000). Therefore, one neuron can receive synaptic connections from thousands of axon terminals. They receive signals or APs from other neurons and propagate to the target neurons via the axon. Basically, processes from axon are pre-synaptic terminals and dendrites serve as post-synaptic contacts for these terminals via synaptic cleft.

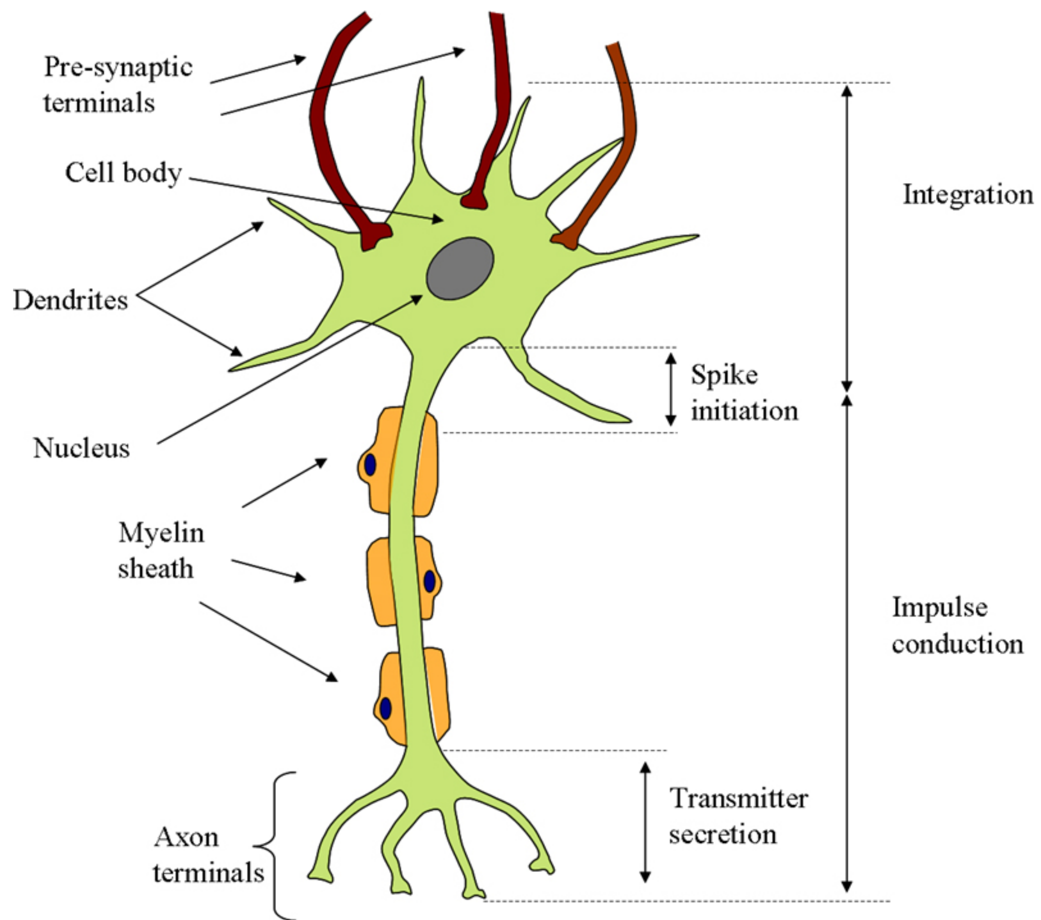


Figure 1.1: Basic morphology of neuron.

Neuron showing the cell body, dendrites, axon and axon (pre-synaptic) terminals with myelin sheath. Cell body receives action potentials (APs) from pre-synaptic axon terminals and gets transmitted via axon to neighbouring neuron.

1.1.2 Glial cell

Glial cells or glia are the predominant cells of the nervous system. Literally, glia means 'glue' in Greek but it does not hold or glue the neurons to each other. Regarding the number of glial cells in the brain, it is still not clear although studies have claimed that glia outnumber neurons by 10 to 50 fold (Kandel, 2000; Hilgetag and Barbas, 2009). However, some studies in rhesus monkeys have shown glia-neuron ratio to be 0.82 (Lidow and Song, 2001) and 0.58 (Christensen *et al.*, 2007). Further, it has been suggested that the myth of 10 to 15 folds more glia has still to be validated (Hilgetag and Barbas, 2009). These cells have a diverse range of functions, with some cells involved in supporting neurons to establish the definite architecture of the brain while others insulate the axons, helping in speeding the signalling processes of the neurons. Previously, it was thought that they are not directly involved in signal transfer. However, it has been demonstrated that they can modulate the signalling process via calcium release of gliotransmitters (Wang *et al.*, 2006; Halassa *et al.*, 2007; Santello and Volterra, 2009; Allaman *et al.*, 2011) and also play a vital role in synapse formation (Eroglu and Barres 2010). These cells are involved in the regulation of synaptic strength, signals and neurotransmitter modulation, synaptogenesis, neurogenesis and metabolic signalling cascades (Nedergaard *et al.*, 2003; Winship *et al.*, 2007; Allaman *et al.*, 2011). Glial cells are divided into two major classes; macroglia and microglia.

1.1.2.1 Macroglia

There are three types of macroglia which comprises of oligodendrocytes, Schwann cells and astrocytes.

1.1.2.1.1 Oligodendrocytes and Schwann cells

Oligodendrocytes and Schwann cells form a myelin sheath which insulates the axon. In the CNS, oligodendrocytes insulate the axon whereas in the peripheral nervous system (Figure 1.2), Schwann cells form the myelin sheath (Figure 1.1). A myelin sheath is

composed of high lipid content, mainly cerebroside, cholesterol, ethanolamine-containing plasmalogens and lecithin. The protein content of a myelin sheath is primarily formed by proteolipid protein (PLP) and myelin basic protein (MBP). Other proteins related to myelin are myelin-oligodendrocyte protein (MOG), oligodendrocyte-myelin protein (OMgP), myelin-associated protein (MAG) and 2', 3'-cyclo nucleotide 3'-phosphodiasterase (CNP) (Pfeiffer *et al.*, 1993).

Oligodendrocytes are not restricted in forming myelin sheath to one axon but covering several axons with myelin (Pfeiffer *et al.*, 1993). Usually, oligodendrocytes forming myelin sheaths of axons with large diameter have fewer processes. These cells express proteins that are responsible for forming gap junctions, connexin29, connexin32, connexin45 and connexin47 which facilitate direct free diffusion of small molecules between two cells (Nagy and Rash, 2000; Orthmann-Murphy *et al.*, 2008). Connexins which are present in each layer of myelin sheath can help in transmitting signals from axons to oligodendrocytes. These proteins are critical for normal myelin formation in the CNS and it is suggested that animal lacking connexins cannot survive (Menichella *et al.*, 2003). This study demonstrated that animal lacking connexins died 6 weeks after birth, with myelin abnormalities leading to thin or absent of myelin sheaths, vacuolation, oligodendrocyte death and axonal loss. Oligodendrocytes do not form myelin sheaths in all neurons but function and physiology of each neuron decide which neurons are destined for myelination. Neurons having larger diameter as well as longer axons such as those being involved in motor function are first to be myelinated during early development (Duncan, 1934; Aguayo *et al.*, 1976). The larger diameter of an axon also determines the thickness of the myelin sheath, the larger the diameter, the thicker the myelin sheath. It has also been shown that survival of oligodendrocytes depends upon the presence of axon and axon-dependent signals help in determining the number of oligodendrocyte required for the myelination (Barres and Raff, 1993; Stevens *et al.*, 1998).

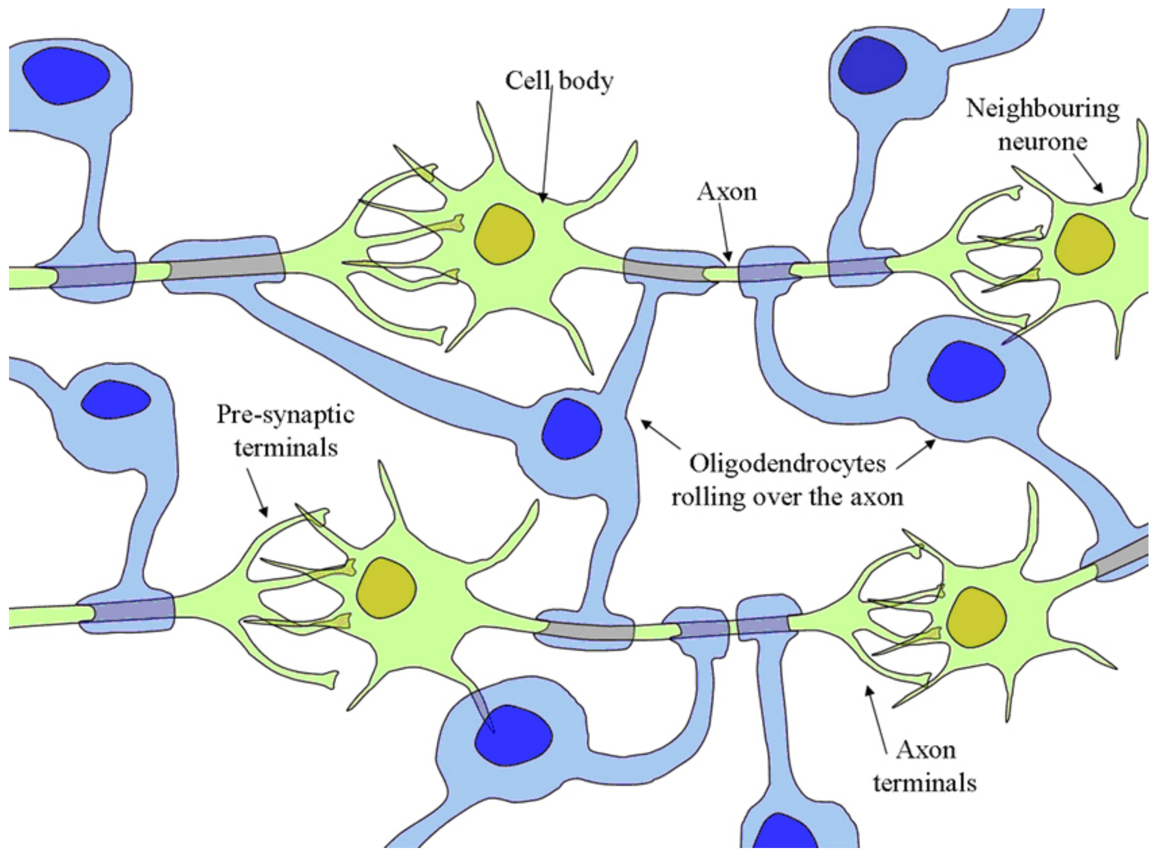


Figure 1.2: Schematic figure of oligodendrocytes insulating axons.

Oligodendrocytes form myelin sheaths in the CNS. These glial cells roll over axons and insulate them. This insulation helps in transmission of signals to different parts of the body. Such signal transmission is known as saltatory conduction.

Under certain pathological conditions, there will be a disturbance in myelin formation. Basically, two types of disorders are associated with myelin sheath, demyelination and dysmyelination. In demyelination, there is a breakdown of the normal myelin sheath along with oligodendrocytes. The most common demyelinating disease of the CNS is multiple sclerosis (MS), an autoimmune disease, characterized by neurological deficit including motor functions (Noseworthy *et al.*, 2000; Hemmer *et al.*, 2002; Peterson and Fujinami, 2007; Comabella and Khoury, 2011). Conversely, in dysmyelination (also referred as hypomyelination), the normal myelin sheath will never form in required quantities during nervous system development. This is often linked to a genetic disorder of mice with point mutation in the gene encoding PLP, which is pathologically and genetically similar to X-linked Pelizaeus–Merzbacher disease (Hudson *et al.*, 1989; Trofatter *et al.*, 1989).

1.1.2.1.2 Astrocytes

Astrocytes are star-shaped cells and are predominant in the CNS glial cell population. These cells are interwoven with neurons and non-neuronal cells in the CNS, filling the space between neurons. They also form a tight junction of the blood-brain barrier (BBB) along with the endothelial cell lining of the blood vessels (Figure 1.3) (Wolburg *et al.*, 1994; Abbott *et al.*, 2006; Persidsky *et al.*, 2006; Bernacki *et al.*, 2008) and provide a micro-architecture of grey matter having separate domains, referred to as, 'astrocytic domains' (Bushong *et al.*, 2002; Bushong *et al.*, 2004; Allaman *et al.*, 2011). The 'astrocytic domain' of one astrocyte rarely overlaps with another (Bushong *et al.*, 2002; Bushong *et al.*, 2004). The processes from astrocytes envelop about four neuronal bodies on average and more than a hundred dendrites (Halassa *et al.*, 2007). Therefore, it has been suggested that signals from a single astrocyte can affect hundreds of neurons.

Under normal physiological conditions, astrocytes play a major role in maintaining ionic composition, extracellular osmolarity, pH and clearing excess neurotransmitters from the synaptic cleft (Anderson and Swanson, 2000; Matsuura *et al.*, 2002; Sofroniew and

Vinters, 2010). They buffer excess potassium ions from the synaptic cleft (Orkand *et al.*, 1966; Holthoff and Witte, 2000; Kofuji and Newman, 2004) as an increase in extracellular potassium ions can alter neuronal membrane potential leading to hyperexcitability of neurons compromising CNS function (Walz, 2000; Belanger and Magistretti, 2009). Astrocytes express different types of potassium (K^+) channels including Ca^{2+}-activated K^+ and voltage-dependent K^+ channels (Roy *et al.*, 1996; Price *et al.*, 2002). These K^+ channels in astrocytes help to regulate the blood flow in the CNS micro-vasculature (Filosa *et al.*, 2004; Koehler *et al.*, 2009). The gap junctions present between astrocytes and oligodendrocytes help in K^+ buffering (Orthmann-Murphy *et al.*, 2008) and maintain K^+ homeostasis in the CNS (Kozoriz *et al.*, 2006; Meeks and Mennerick, 2007; Ostby *et al.*, 2009). Similarly, a small alteration in pH can affect neuronal functions including energy metabolism, membrane excitability and synaptic transmission (Deitmer and Rose, 1996; Obara *et al.*, 2008) and astrocyte act as a carbon dioxide (CO_2) sink by converting CO_2 to H^+ and $HCO3^-$ with the help of carbonic anhydrase expressed in astrocytes (Cammer and Tasey, 1988; Belanger and Magistretti, 2009). Further, as excess accumulation of glutamate at synaptic cleft exert neurotoxicity (Danbolt, 2001; Sattler and Tymianski, 2001), astrocytes uptake excess glutamate from the synaptic cleft to protect neurons from the glutamate toxicity (Danbolt, 2001; Gadea and López-Colomé, 2001). In addition, astrocytes also provide sufficient energy substrates such as glucose and lactate to neurons when extracellular levels of these substrates are low at the synaptic cleft (Rouach *et al.*, 2008) along with growth factors (Martin, 1992; Sokoloff *et al.*, 1996; Kintner *et al.*, 2004).

Astrocytes further play an important role in the regulation of neuronal function by integrating neuronal firing and synaptic networks via their cytoplasmic calcium (Ca^{2+}) waves (Dani *et al.*, 1992; Pasti *et al.*, 1997; Santello and Volterra, 2009). The intracellular calcium level in astrocytes increases in response to neuronal activity triggered by stimulation (Wang *et al.*, 2006; Winship *et al.*, 2007). These calcium waves generated can be propagated to their neighbouring astrocytes via gap junctions (Boitano *et al.*, 1992; Sanderson *et al.*, 1994) as well as extracellular medium (Guthrie *et al.*,

1999) and can travel long distances from the originated site. These calcium signals cause the release of glio-transmitters including glutamate, adenosine triphosphate (ATP) and D-serine (Yang *et al.*, 2003; Fellin *et al.*, 2004; Gordon *et al.*, 2005; Halassa *et al.*, 2007; Belanger and Magistretti 2009) which can modulate synaptic transmission between neurons (Kang *et al.*, 1998; Halassa *et al.*, 2007; Di Castro *et al.*, 2011). It has been shown that the stimulation of astrocytes increases inhibitory post-synaptic currents in pyramidal neurons of hippocampus (Kang *et al.*, 1998). In addition, glutamate released from astrocytes can directly influence neuronal activities and synaptic plasticity (Angulo *et al.*, 2004). It has also been suggested that by regulating synaptic transmission, astrocytes also can control synaptic plasticity (Pascual *et al.*, 2005; Panatier *et al.*, 2006; Santello and Volterra, 2009).

On the other hand, a role for astrocytes have been implicated in various neurodegenerative diseases including Alzheimer's disease (AD), MS, Parkinson's disease (PD) and stroke (Seifert *et al.*, 2006; Nair *et al.*, 2008; Rodriguez *et al.*, 2008; Allaman *et al.*, 2010; Chao *et al.*, 2010; Gu *et al.*, 2010). Under such pathological conditions, astrocytes become activated (reactive astrogliosis) (Sofroniew and Vinters, 2010) which are characterised by altered gene expression, hypertrophy and proliferation (Ridet *et al.*, 1997). Activated astrocytes release various cytokines, chemokines, reactive oxygen species and growth factors (Sofroniew, 2005; Farina *et al.*, 2007). Pathological conditions in the CNS can compromise the intrinsic anti-oxidative property as well as glutamate clearance property of astrocytes causing neuronal death (Matsuura *et al.*, 2002; Garg *et al.*, 2008; Allaman *et al.*, 2011).

1.1.2.2 Microglia

Microglial cells are considered as the macrophages of the CNS and are the immunocompetent cell of the CNS (Kreutzberg, 1996; Streit *et al.*, 1999). It has been suggested that these cells have heterogeneous morphology (which varies with the region of the brain) and are classified into three different groups; compact cells, longitudinally

branched cells and radially branched cells (Lawson *et al.*, 1990). Though microglia can be found everywhere in the CNS, distribution patterns of these cells are not homogenous (Perry *et al.*, 1985; Lawson *et al.*, 1990). These cells reside in the choroid plexus, ventricles, perivascular spaces, meninges, grey matter and white matter of the CNS (Perry *et al.*, 1985; Pow *et al.*, 1989; Lawson *et al.*, 1990; Ransohoff and Cardona, 2010). It has been reported that microglial cells are more efficient in antigen presentation in comparison to astrocytes (Aloisi *et al.*, 1998). These cells are presumably derived from circulating pool of monocytes and can act as an antigen presenting cell in the CNS (Hickey and Kimura, 1988; Lawson *et al.*, 1990; Hickey, 2001; Ransohoff and Perry, 2009; Ransohoff and Cardona, 2010) and are found to be regularly replaced from the bone marrow (Hickey and Kimura, 1988; Priller *et al.*, 2006). Microglial cells are characterised into two phenotypes on the basis of their response to different cytokines (Michelucci *et al.*, 2009; Fenn *et al.*, In press). IFN-γ induces M1-like phenotypes while stimulation of microglia by IL-10 or IL-4 gives rise to M2-like phenotypes. M1-like phenotypes are responsible for producing pro-inflmmatory cytokines whereas M2-like phenotypes show anti-inflmmatory response as well as helps in tissue regeneration (Mantovani *et al.*, 2002; Michelucci *et al.*, 2009).

Under normal conditions, microglia have a small cell body with a number of fine, branched processes (Ransohoff and Perry, 2009) which are constantly monitoring their environment for the normal physiological response and are capable of responding to abnormal changes or disturbances (Kreutzberg, 1996; Stoll *et al.*, 1998; Streit *et al.*, 1999; Hanisch and Kettenmann, 2007). These cells have the ability to sense and respond to any danger signals or CNS insults or injury by removing dead neurons from the injured site (Kreutzberg, 1996). In the CNS, microglial cells remain in a 'stand-by mode' not as a resting stage (van Rossum and Hanisch, 2004; Hanisch and Kettenmann, 2007). They respond to any danger signal in 'on' and 'off' mode and will be in ready position to respond to any changes in the CNS. Multi-photon live imaging of these cells has revealed that their fine processes are highly mobile (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Time-lapse imaging has shown continuous extension and

retraction of the processes over tine, and it is estimated that the brain parenchyma will be monitored every few hours (Davalos *et al.*, 2005), assessing the condition of the CNS micro-environment for unusual concentration or altered features of soluble and insoluble chemicals which may be the informer about danger signals (Honda *et al.*, 2001; Hoffmann *et al.*, 2003; van Rossum and Hanisch, 2004).

Under pathological conditions (including neurodegenerative diseases, traumatic injuries, and stroke) microglial cells become activated and form amoeboid structures from ramified phenotypes (Kreutzberg, 1996; Fetler and Amigorena, 2005; Heneka *et al.*, 2010). In the response to any CNS injuries or insults, they get activated and these activated microglial cells can damage the healthy neurons by releasing different inflammatory mediators including cytokines, reactive oxygen species, nitric oxide, neurotoxic secretary products and free radical species (Griffin *et al.*, 1998; Appel and Simpson, 2001; Heneka and O'Banion, 2007). These factors induce glutamate excitotoxicity either by releasing more glutamate or by increasing susceptibility to glutamate receptor activation, and leading to neuronal death (Appel and Simpson, 2001; Zhao *et al.*, 2004; Appel *et al.*, 2009). Moreover, these cells are found to be actively involved in number of neurodegenerative diseases including Alzheimer’s disease (AD) (Heneka *et al.*, 2010), amyotrophic lateral sclerosis (ALS) (Zhao *et al.*, 2004), multiple sclerosis (MS) (Napoli and Neumann, 2009) and Parkinson’s disease (PD) (Appel *et al.*, 2009).

1.2 The brain, an immune-privileged site?

Whether or not the brain is an immune-privileged site has been an intense debate among neuroscientists. The concept of an immuno-privileged site was first highlighted when allografts in eye and brain tissue were better persisted in comparison to other peripheral tissues (Billingham and Boswell, 1953; Barker and Billingham, 1977). These studies have demonstrated that the graft hardly showed any rejection in comparison to other parts of the body. On the other hand, proper neuronal function demands a tight

regulation of the CNS microenvironment including concentration of different ions and other noxious substances(Hawkins and Davis, 2005). For this proper control and the normal function of the CNS, the BBB plays an important role (Weiss *et al.*, 2009).

The BBB lies at the interface of the blood and the brain tissue that comprises microvascular endothelium, astrocytes, basement-membrane and pericytes (Wolburg and Lippoldt, 2002; Persidsky *et al.*, 2006). To address the collective terms for the constituents of the BBB, the concept of a 'neurovascular unit' was proposed to emphasize more for the structure and function of BBB integrity (Hawkins and Davis, 2005; Weiss *et al.*, 2009). This unit protects the CNS from fluctuations of nutrients, hormones, metabolites and blood constituents including endogenous and exogenous compounds (Hawkins and Davis, 2005; Bernacki *et al.*, 2008). The endothelial cells of the BBB have distinct morphological and physiological properties in comparison to other endothelial cells of the body (Abbott, 2005; Persidsky *et al.*, 2006; Weiss *et al.*, 2009). These endothelial cells lack fenestration with a tight junction reducing permeability to intercellular pathways and they express very low numbers of non-specific transporters (Petty and Lo, 2002; Abbott, 2005; Persidsky *et al.*, 2006). Moreover, the highly polarized receptors and transporter systems play an important role in movement of nutrients into the CNS and efflux of metabolites or toxic substances into the vascular compartment (Brightman and Kadota, 1992; Petty and Lo, 2002; Weiss *et al.*, 2009). Furthermore, it has also been suggested that pericytes play a major role in maintaining the BBB tight junction along with these endothelial cells (Balabanov and Dore-Duffy, 1998; Armulik *et al.*, 2010). An *in vivo* imaging study of mice lacking pericytes showed increase in permeability of the BBB to water and low-molecular and high-molecular mass tracers (Armulik *et al.*, 2010).

Hence, the presence of a neurovascular unit, lack of conventional lymphatic vessels and limited parenchymal expression of major histocompatibility complex (MHC) molecules in the CNS (Engelhardt, 2006) along with the limited rejection of allografts in the CNS might have led us to consider the CNS as a immunologically privileged area. However,

it is now well accepted that the immunologically privileged brain is no longer considered immune-privileged. There is increasing evidence for the regular communication between the CNS and the immune system (Straub *et al.*, 1998; Bucky Jones *et al.*, 2007; Kerschensteiner *et al.*, 2009). Research has shown that immune cells can enter the CNS via the choroid-plexus and reside in perivascular spaces or move into the neuropil (Hickey 1991; Weller *et al.*, 1996; Carson *et al.*, 2006; Reboldi *et al.*, 2009). It has been shown that the extravasation of these immune cells into the CNS involves a very complex set of adhesion molecules present on the endothelial cells and on the cell surface of these immune cells (Hickey *et al.*, 1991; Laschinger and Engelhardt, 2000; Reboldi *et al.*, 2009). Common adhesion molecules that have been suggested to be involved in the extravasation of leukocytes include the intracellular adhesion molecule (ICAM) family, vascular cell adhesion molecule (VCAM), integrins, selectins and the junctional adhesion molecule (JAM) family (Springer, 1994; Wolburg and Lippoldt, 2002; Engelhardt and Wolburg, 2004; Engelhardt, 2006; Engelhardt, 2010). Hence, this evidence suggests that the CNS is not an immunologically privileged area. Nevertheless, it is also true that the CNS is a more immunologically privileged site relative to other organs due to the presence of the BBB and tightly regulated immune response including limited movement of immune cells and expression of death ligand (Galea *et al.*, 2007; Weiss *et al.*, 2009).

1.3 Basic immunology

The immune-system is a complex, organized system to respond against any insult to the body, such as resisting any foreign particles or chemicals, or any invading micro-organisms. The initial inflammatory response to danger signals evoked by infection or irritation results in accumulation of fluid and plasma proteins along with leukocytes in the affected part (Perry *et al.*, 1993; Abbas *et al.*, 2010). In the CNS, acute inflammatory response can be seen during infections and other acute injuries (Andersson *et al.*, 1992; Carlson *et al.*, 1998) whereas chronic inflammatory response is a characteristic of

neurodegenerative diseases including AD, PD and MS (Hemmer *et al.*, 2002; Hunot and Hirsch, 2003; Heneka and O'Banion, 2007).

Immune responses are mediated by leukocytes (or white blood cells), which originate from bone marrow and mature in the bone marrow, thymus or periphery. All cells arise from pluripotent hematopoietic stem cells, which differentiate into either common lymphoid progenitor cells or common myeloid progenitor cells. The common myeloid progenitor cells give rise to either granulocyte/macrophage progenitor cells or megakaryocyte/erythrocyte progenitor cells, differentiating into platelets and red blood cells (erythrocytes) as well as neutrophils, eosinophils, basophils, monocytes, macrophages, dendritic cells and mast cells. These myeloid-derived cells are mainly involved in the innate immune responses (Janeway and Medzhitov, 2002; Murphy *et al.*, 2008). Innate immunity is the first line of defence for the immune system, and exists in every living being, including plants, to protect from any infections. It is an initial response to any pathogens to protect the host from any harmful effects and it also alerts adaptive immune responses to the presence of pathogens by presenting antigens (Fearon and Locksley, 1996). However, the innate immune system has a limited diversity in recognising different pathogens (Abbas *et al.*, 2010) and is relatively restricted to a number of pathogen-associated molecular patterns (Fearon and Locksley, 1996; Abbas *et al.*, 2010). Different types of micro-organisms express defined pathogen-associated molecular patterns such as peptidoglycan, lipopolysaccharides, bacterial lipoproteins and bacterial carbohydrates and receptors for these antigens are referred to as pattern recognition receptors (Janeway and Medzhitov, 2002; Akira *et al.*, 2006; Heeg *et al.*, 2007). Most of the cells involved in innate response are phagocytes including macrophages and neutrophils. Macrophages and neutrophils phagocytose bacteria and other pathogens, whereas other myeloid-derived cells such as mast cells, eosinophils and basophils are involved in initiating the inflammatory response to alert adaptive immune system by which the body deals with insults from physical or chemical agents and microbial invasion (Kaufmann and Steward, 2005).

Although the innate immune response plays a critical role in the first line of host defence system, it has limitations in recognising the wide range of pathogenic molecular patterns (Bonilla and Oettgen, 2010). Therefore, the adaptive immune system has evolved to protect from a wide antigenic repertoire, as well as to counteract the potential for pathogens to overcome host defense system by mutation (Cooper and Alder, 2006; Bonilla and Oettgen, 2010). In the innate immune system, the recognition receptors are encoded in their functional form in the germline genome while receptors of the adaptive immune system are custom tailored and selected through a somatic recombination of a large array of gene segments (Bonilla and Oettgen, 2010). In the adaptive immunity, a group of cells called lymphocytes are involved. There are mainly two types of lymphocytes, B cells and T cells, which arise from common lymphoid progenitor cells and develop either in the bone marrow or the thymus. B cells arise from the bone marrow whereas T cells develop and differentiate in the thymus. These lymphocytes exit from either bone marrow or thymus move to secondary lymphoid organs including lymph nodes and spleen, through which they continue to circulate until they encounter their cognate antigen (Bonilla and Oettgen, 2010). The adaptive immune response initiated following signals from innate immune response either via antigens directly or indirectly through antigen presenting cells (Fearon and Locksley, 1996) (Figure 1.3). At this point, dendritic cells which are effective antigen presenting cells (Banchereau and Steinman, 1998) play a pivotal role for the effective immune response. In this way, these cells act as a bridge between innate immunity and adaptive immunity. During this process, dendritic cells take up various types of antigens and processed which finally presented to lymphocytes especially T cells (Mellman and Steinman, 2001) (Figure 1.3). These interactions provide specific recognition of antigens and immunological memory of these antigens contributing in long-lasting protection against these antigens (Abbas *et al.*, 2010). These lymphocytes then exit from lymph nodes and spleen which migrate to different parts of the body to initiate immune response (Bonilla and Oettgen, 2010).

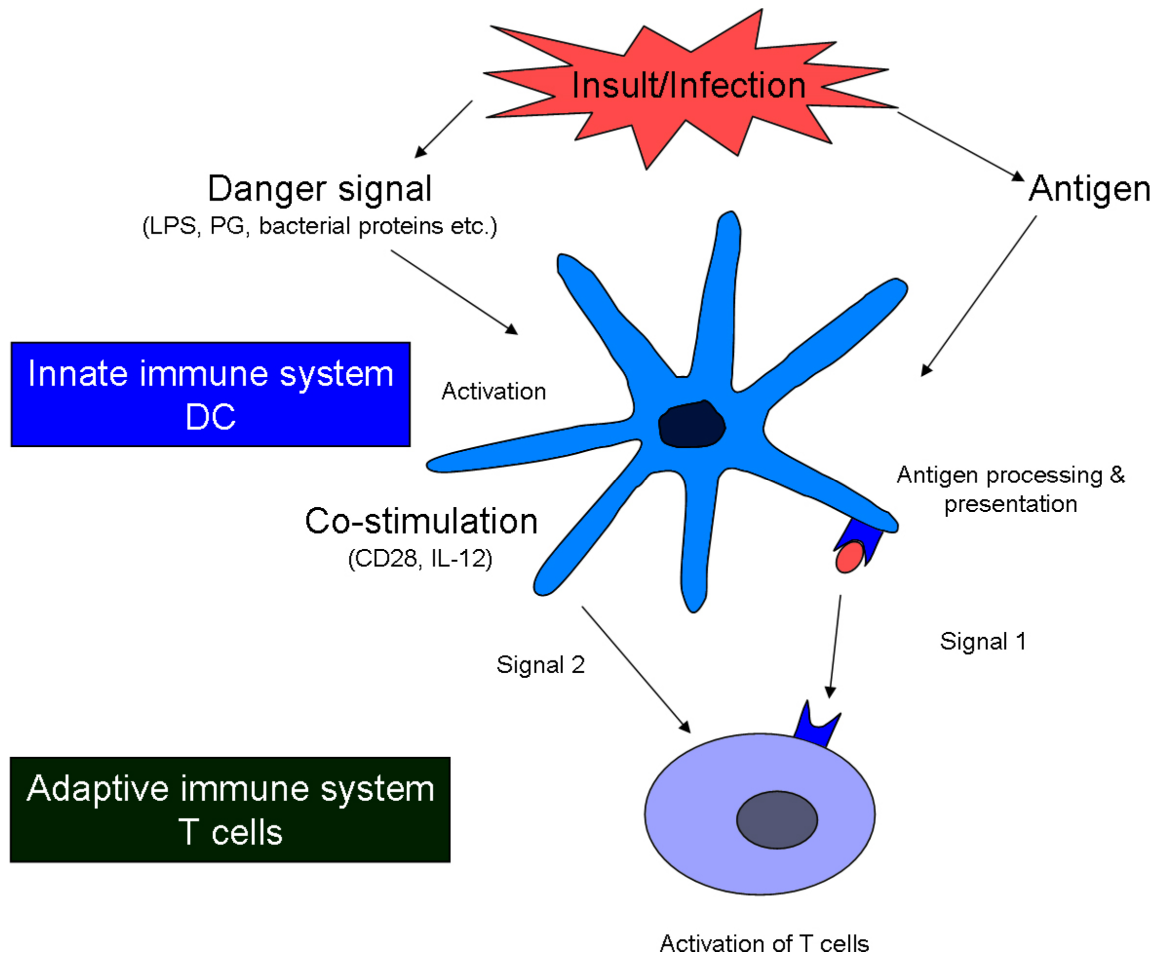


Figure 1.3 Steps in activation of the adaptive immune system by the innate immune system.

The innate immune system is the first line defense system to protect the host from any harmful effects. Thus, following any insults or infections, innate immune system rapidly initiates inflammatory response that can alert adaptive immune system. These two systems interact with each other either via direct cell contact or chemical mediators such as cytokines, chemokines and antibodies. LPS: lipopolysaccharides; PG: proteoglycan; DC: dendritic cell. (Adapted from Heeg *et al.*, 2007).

1.3.1 T cells

T cells are one of the major components of the adaptive immune system and play a critical role in acquired immunity. They serve as the effector cells through antigen specific cytotoxic activity or by releasing inflammatory mediators like cytokines (Gordon, 2003). There are mainly two types of T cells identified; $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. These two types of T cells are differentiated by the specific type of MHC complex they recognize, with $CD4^+$ T cells recognising antigens presented by the MHC class II complex and $CD8^+$ T cells restricted to MHC class I associated antigens (Guermonprez *et al.*, 2002). $CD4^+$ T cells help co-stimulate B cell activation and antibody secretion, macrophage activation, cytokine production and help effective $CD8^+$ T cell activation (Gordon, 2003). Conversely, $CD8^+$ T cells can be directly involved in clearing out viruses as well as killing infected cells (York and Rock, 1996) and are also referred to as cytotoxic T cells (Dittel, 2008).

Both $CD4^+$ and $CD8^+$ T cells recognise peptide antigens presented in the context of MHC I and II respectively. Antigens are degraded into short peptides and loaded into MHC inside antigen-presenting cells. Exogenous and endogenous antigens are processed via endocytosis and loaded to MHC II while only endogenous antigens are loaded into MHC I in the endoplasmic reticulum (Guermonprez *et al.*, 2002). These processed antigens are presented to specific T cells via a cell surface receptor which is referred as a T cell receptor (TCR). A T cell receptor is similar to the immunoglobulin and has two peptide chains - an α and β chain, along with other protein components, including CD3, which together form the TCR complex. The CD3 complex includes four distinct chains (CD3γ, CD3δ, CD3ε and CD3 ζ; Figure 1.4) and is involved in the activation of signals in T cells (Abbas and Lichtman, 2003). For optimal signalling through the TCR complex, the co-receptors CD4 or CD8 must also bind to nonpolymorphic regions of their respective MHC molecule (Veillette *et al.*, 1988; Barber *et al.*, 1989; Sakihama *et al.*, 1995). Therefore, CD4 can bind to MHC II while CD8 can bind to MHC I only as mentioned earlier. Such specific interaction of these receptors is referred to as a MHC restriction.

Recognition of antigen is a key step in T cell activation and there is a fundamental role for antigen presenting cells (APCs). APCs engulf pathogens or dying cells and can generate antigenic peptides which are loaded onto the MHC molecules and presented on the cell surface (Unanue, 1984; Guermonprez *et al.*, 2002). Both naïve $CD4^+$ and $CD8^+$ T cells are activated following recognition of their specific antigen/MHC complex, but require co-stimulation for optimal activation (Shaw and Dustin, 1997). Thus, primary immune responses are only generated following activation by APCs expressing appropriate co-stimulatory molecules including CD28, CD80, CD86 and integrins (Croft, 1994; Dustin *et al.*, 1996), and these are referred to as 'professional' APCs. Conversely, 'primed' T cells (those T cells which have already been fully activated following an encounter with antigen/MHC) (Croft, 1994) are less dependent on co-stimulation. Thus, 'non-professional' APCs in the CNS like astrocytes and endothelial cells (Croft, 1994; Williams *et al.*, 1995; Becher *et al.*, 2000) can only initiate secondary responses (Matsumoto *et al.*, 1992; Becher *et al.*, 2000) and for full activation T cells need co-stimulation (Matsumoto *et al.*, 1992; Williams *et al.*, 1995).

Although responsible for recognition of antigen/MHC, the TCR itself cannot initiate intracellular signalling because of short cytoplasmic regions. The multimeric complex, CD3 is critical for effective signal transduction following ligation of the TCR (Sussman *et al.*, 1988; Hall *et al.*, 1991). Additionally, co-stimulatory molecules like CD28 and inducible T cell co-stimulator (ICOS) are also essential for the effective T cell activation (Sharpe and Freeman, 2002). Sustained interaction between TCR and specific antigen/MHC molecules is required for effective signal transduction and this is achieved by formation of supramoleculecular activation clusters (Grakoui *et al.*, 1999; Bromley *et al.*, 2001). This supramolecular activation clusters comprise the TCR, co-receptors, co-stimulatory, adhesion receptors and signalling molecules. The formation of supramolecular activation clusters after interaction between T cell and APCs is also referred as the 'immunological synapse (IS)' (Monks *et al.*, 1998; Grakoui *et al.*, 1999). The IS formation is between T cells and APCs has TCR-rich central supramolecular

activation clusters surrounded by integrin-rich peripheral supramolecular activation clusters (Monks *et al.*, 1998; Smith-Garvin *et al.*, 2009). This interaction leads to proliferation and activation of T cells. These activated T cells differentiated into various phenotypically and functionally different effector T cells including cytotoxic T cells, Th-1 cells, Th-2 cells, Th-17 cells and many more subtypes (Mosmann *et al.*, 1986; Guermonprez *et al.*, 2002; Dittel, 2008; Wan and Flavell, 2009).

The differentiation of $CD4^+$ T cells (T helper cells) into various effector T cells is induced by cytokines produced by activated cells of innate and adaptive immune system (Wan and Flavell, 2009). Naïve T helper cells can differentiate into T helper (Th)-1 cells if stimulated by interleukin (IL)-12, IL-18 and/or IL-27 or interferon (IFN)-γ (Abbas *et al.*, 1996). Th-1 cells release cytokines like TNF-α and IFN-γ which are considered as signature cytokines. Th-1 cells are involved in the activation of macrophages as well as these cells increase the cytotoxic activity of natural killer (NK) cells and CD8+ cells (Abbas *et al.*, 1996; Gordon, 2003). Conversely, IL-4 induced Th-2 differentiation is associated with the signature cytokines IL-4, IL-5, IL-10, IL-13 (Abbas *et al.*, 1996). These cells are responsible for directing B cells to release antibodies against antigens and also involved in inhibition of inflammatory Th-1 responses (Abbas *et al.*, 1996; Romagnani, 2006; Dittel, 2008). In addition to these two helper cells, other subsets of T helper cells have recently been described: Th-17 cells, regulatory T cells (T_{reg}), follicular helper T cells and Th-9 cells. IL-23 or IL-6 along with transforming growth factor (TGF)-β stimulated naive T helper cells differentiate into pathogenic Th-17 cells which produce IL-17, IL-6 IL-21, IL-22 (Langrish *et al.*, 2005; Mangan *et al.*, 2006; Korn *et al.*, 2007; Zheng *et al.*, 2007). Moreover, T_{reg} cells are produced when naïve T helper cells are stimulated by TGF-β and IL-10 or IL-2 (Nakamura *et al.*, 2001; Chen *et al.*, 2003; Bettelli *et al.*, 2006) and can secrete IL-10 and TGF-β (Weiner, 2001; Stassen *et al.*, 2004). Normally, T_{reg} cells modulate the inflammatory responses by the production of inhibitory cytokines (Scheffold *et al.*, 2007; Dittel, 2008). When naïve T helper cells are activated in the context of IL-6 or IL-21, these cells can differentiate into IL-21-producing follicular helper T (Tfh) cells whose role is to help in production of antibodies

by B cells (Chtanova *et al.*, 2004; Vogelzang *et al.*, 2008; Crotty, 2011). Similarly, the newly identified Th-9 cells are IL-4 and TGF-β driven T helper cells differentiation and are responsible for IL-9 secretion, which has been suggested to be involved in the initiation of inflammation (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008).

Inflammatory signals induce the expression of chemokine receptors on the activated T cells that help in migration to the B cell zone within the secondary lymphoid organ to interact with antigen-specific B cells and provide help for antibody production (Jenkins *et al.*, 2001). Normally, most of the activated T cells die within the lymphoid organs but in the presence of inflammation two populations of memory T cells survive (Lenardo *et al.*, 1999; Jenkins *et al.*, 2001). One population of these cells recirculate through non-lymphoid tissues while other recirculates through lymph nodes and these cells can initiate immediate immune response on the encounter to their cognate antigens (Dutton *et al.*, 1998; Jenkins *et al.*, 2001). Following encounter with APCs, activated T cells exit from the lymph node via high endothelial venules which have the ability to enter peripheral tissues and recognise their cognate antigens presented by non-professional APCs. Once these T cells find their cognate antigens, they initiate immune response and release various cytokines and chemokines at the site which act as a chemoattractant allowing more immune cells including lymphocytes, neutrophils and monocytes migration to the inflammatory site including CNS (Tani and Ransohoff, 1994; Ransohoff *et al.*, 1996; Rot and von Andrian, 2004). In addition, in response to inflammation vascular endothelial cells also express adhesion molecules which help in migration of activated T cells to other non-lymphoid tissues including the CNS (van Oosten *et al.*, 1995; Jenkins *et al.*, 2001; Pedemonte *et al.*, 2006). Unlike naïve T cells, the activated T cells express specific chemokine receptors, integrins and selectins which enable them to interact with adhesion molecules expressed in the vascular endothelium during migration (Engelhardt *et al.*, 1994; Jenkins *et al.*, 2001; Engelhardt, 2006).

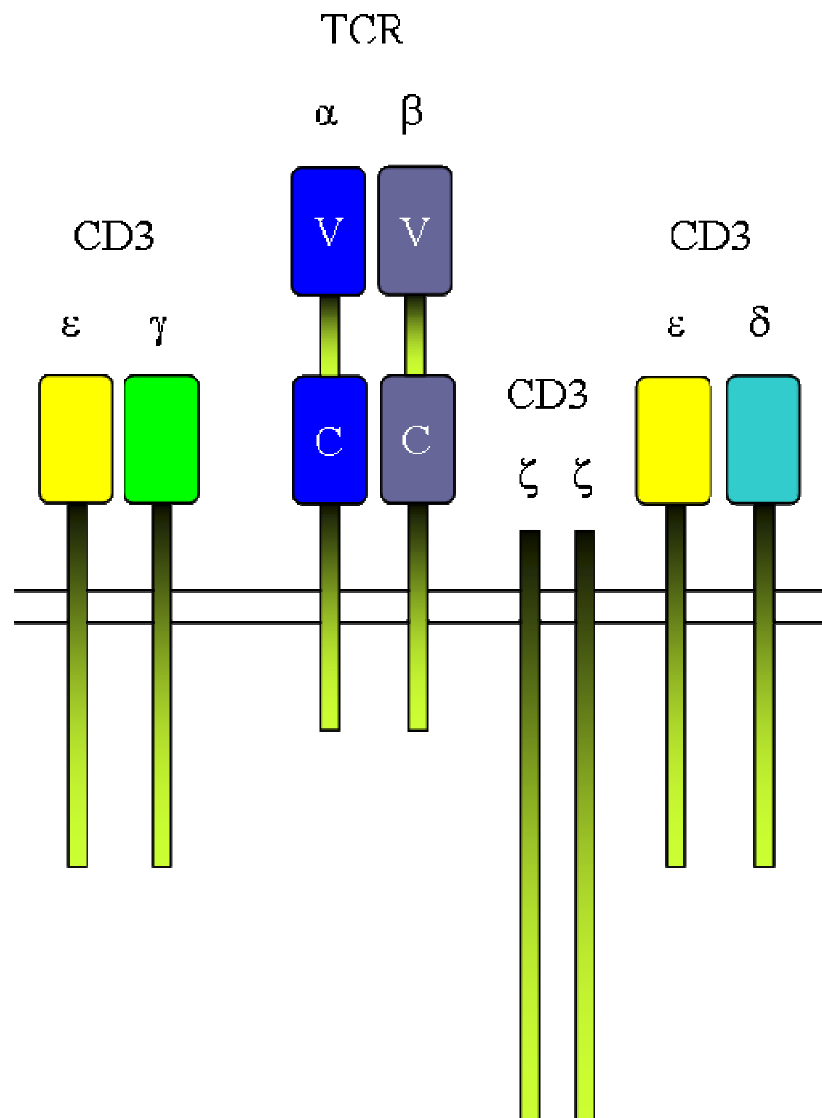


Figure 1.4: T cell receptor (TCR)/CD3 complex.

TCR has two peptide chains; α and β whereas, CD3 has 4 different subunits; ε, ζ, γ and δ.

1.3.2 B cells

Although B cells were originally identified to mature in the bursa of Fabricius of birds (from where these cells got their name) mammalian B cells mature in the bone marrow and in liver during foetal life (Raff, 1973). The development of B cells from immature B cells to mature B cells depends upon a functional rearrangement of immunoglobulin heavy (H) and light (L) chain genes (Rolink and Melchers, 1996; Hardy *et al.*, 2000). This arrangement of immunoglobulin is determined by the reordering of variable (V), diverse (D) and joining (J) gene segments in H chain or the V and J gene segment in L chain (LeBien and Tedder, 2008). This rearrangement generates the pre-B cell receptor or pre-BCR, an analogous to BCR (Figure 1.5) in mature B cell. The BCR constitutes two heavy polypeptide chains and two light polypeptide chains which are joined by di-sulphide bond. These heavy chains and light chains consist of variable (V) region and constant (C) region. There are many V genes and four functional J 'mini-genes' in light chains. These each multiple V genes combine with each J 'mini-genes' to form different VJ units by somatic recombination (Hozumi and Tonegawa, 1976; Kaufmann and Steward, 2005). Similarly, in heavy chains also there are multiple V genes and J 'mini-genes' but there is one additional mini-gene family called D gene is also present. Hence, in heavy chain two steps of somatic recombination are required for the generation functional genes (Sakano *et al.*, 1980; Kaufmann and Steward, 2005). VJ units arise from first somatic recombination and this unit again combine with D mini-genes to form VDJ genes by second somatic recombination. Therefore, these randomly rearrangements of various mini-genes families together give wide varieties of total genes and proteins as antibodies or BCR. BCR also contributes to the survival of mature B cell and to their cellular differentiation (Lam *et al.*, 1997; Neuberger, 1997; Hardy *et al.*, 2000).

During B cell activation, recognition of antigen by a B cell induces clustering of BCR and phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosine residues (Rawlings and Witte, 1994; Gold, 2002) causing activation of cellular signalling pathways. During this activation process, the antigen-BCR complex is internalised and the antigen processed for presentation on the cell surface in association

with MHC-II protein, for presention to $CD4^+$ T cells or T helper cells (Lanzavecchia, 1985; Batista *et al.*, 2001). Most antigens induce only weak BCR signalling and recognition of these antigens is not sufficient to activate B cells (Baumgarth, 2000). Hence, for the optimal B cell activation, T cells are required and this is referred as T cell-dependent mode of B cell activation. Following initial activation of B cells by antigens, these cells migrate towards the T cell zone of the lymph node, where they can interact and present antigen/MHC-II to $CD4^+$ T cells (Clark and Ledbetter, 1994). At this stage, T cells interact with their cognate antigen/MHC-II presented by B cells, generating a stable interaction for effective T cell and B cell stimulation (Batista *et al.*, 2001; Harwood and Batista, 2010). Following activation, activated B cells differentiate into the antibody-secreting plasma cell and secrete specific antibodies. Those activated B cells which do not differentiate into plasma cells will initiate the formation of a germinal centre in lymphoid tissue (MacLennan, 1994). In the germinal centre, isotype switching occurs which lead to affinity maturation of B cells as well as generation of high affinity memory B cells (McHeyzer-Williams and McHeyzer-Williams, 2005).

However, B cells can also be activated without any help from T helper cells. If an antigen is highly polymeric in nature (such as of repetitive carbohydrate epitope in bacterial cell membrane or repetitive protein determinant in virus), there will be an effective activation of B cells which do not need help from T cells (Bachmann and Kopf, 1999). This activation process is known as T-cell-independent mode of B cell activation. Usually, conventional B cells are activated with the help of T helper cells while B1 cells are capable of activation without the help of T cells (Fagarasan and Honjo, 2000).

Mature B cells which migrate to lymphoid follicles of lymph nodes and spleen only have access to antigens where these cells initiate immune response (Loder *et al.*, 1999). These B cells differentiated into mainly three types of B cell subtypes; B1 cells, marginal zone B cells and follicular B cells (Martin and Kearney, 2001; Cariappa and Pillai, 2002). These cells are responsible for secretion of antibodies during the immune response and are capable of secreting large varieties of antibodies against wide range of antigens that

host can encounter (Loder *et al.*, 1999; Kaufmann and Steward, 2005; Abbas *et al.*, 2010). This is possible due to the unique arrangement of genes in BCR. This diversity is essential in the immune system as innate immune system has limited common pathogenic molecular patterns as described earlier (see section 1.3).

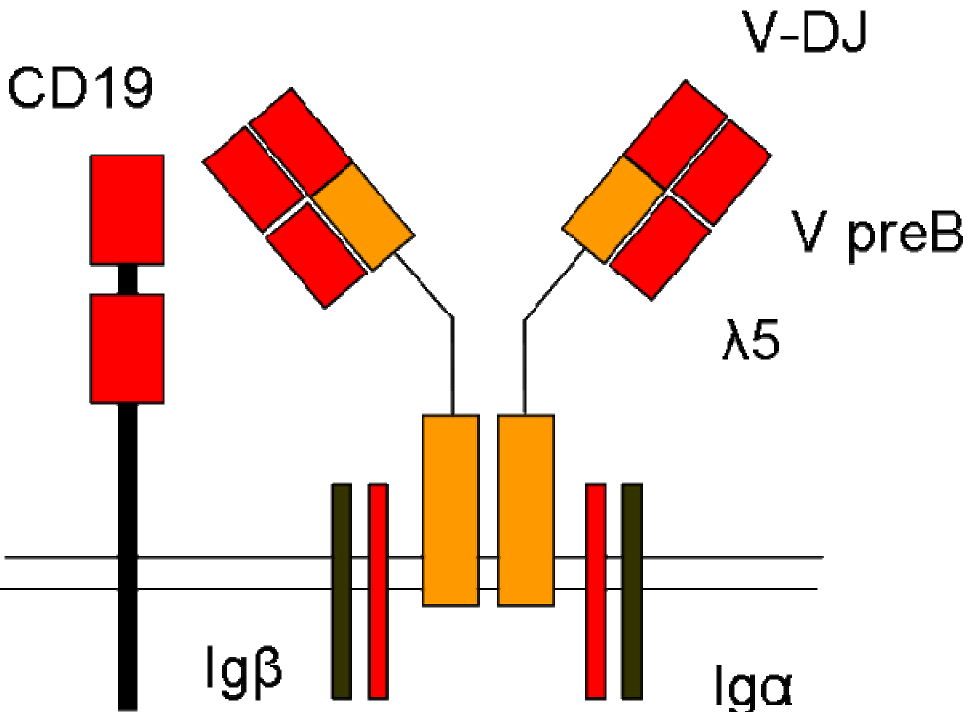


Figure 1.5: B cell receptor complex.

BCR complex showing CD19, BCR along with Igβ and Igα.

1.4 Communication between central nervous system and immune system

1.4.1 Brain modulation of immune function

Initially, the CNS was considered as a functionally autonomous organ but now it has been accepted that the CNS and the immune system have close connections and have a bi-directional neural-immune communication (Madden and Felten, 1995; Jiang *et al.*, 1998; Dantzer, 2004; Besedovsky and Rey, 2007). The most common way of communication is via humoral factors such as cortisol and epinephrine released from the hypothalamic-pituitary-adrenal (HPA) axis (Blalock, 1994; Straub *et al.*, 1998) in response to stress and a stressor is anything that stimulates the activation of HPA axis and sympathetic nervous system (Segerstrom and Miller, 2004; Glaser and Kiecolt-Glaser, 2005). Stress can alter antibody secretions and the release of pro-inflammatory as well as anti-inflammatory mediators (Konstantinos and Sheridan 2001). Most immune cells express receptors for one or more hormones associated with HPA and sympathetic-adrenal-medullary axis (Miller *et al.*, 1998; Welniak *et al.*, 2002; Glaser and Kiecolt-Glaser, 2005) and these hormones can regulate immune function via these receptors such as inhibition of inflammation and shifting production of cytokines from Th-1 cells to Th-2 cells (Elenkov and Chrousos, 2002), trafficking cells from lymph node to peripheral blood (Lai *et al.*, 1998), increasing cytokine production (Radulovic *et al.*, 1999) and maintaining competence of immune cells including T cells, B cells and macrophages (Welniak *et al.*, 2002). It has also been demonstrated that mice fail to recover from experimental autoimmune encephalomyelitis (EAE) after adrenalectomy (MacPhee *et al.*, 1989), which suggests that the HPA axis plays a crucial role in modulation of the immune system. Moreover, nerve fibres present in the lymphoid organs also suggest a bi-directional communication between the CNS and immune system. Recently, it has been demonstrated that stimulation of vagus nerve fibres causes acetylcholine release from spleenic T cells (Rosas-Ballina *et al.*, 2011). These nerve fibres act as a link between these two systems which relay or receive signals and affect the physiological

functions (such as antibody secretion and cytokines and chemokines release) of the immune cells (Felten *et al.*, 1987; Madden and Felten, 1995; Rosas-Ballina *et al.*, 2011).

1.4.2 Immune regulation of brain function

Whilst it is clear that the CNS can influence immunity, the immune system also plays a crucial role in normal brain development, neuronal differentiation and synaptic plasticity (Boulanger and Shatz, 2004; Deverman and Patterson, 2009). Furthermore, various cytokines and chemokines are able to regulate secretion of neurotransmitters in the CNS (Blalock and Smith, 1980; Carlson *et al.*, 1987; Jiang *et al.*, 1998; Dantzer, 2004), with interferons being the first cytokines identified to have an effect on neuronal functions (Smith and Blalock 1981; Blalock, 1994). Cytokines can induce sickness behaviour (characterised by fever, reduction in activity and weight loss) and also regulate neuroendocrine effects, including release of different hormones (Crane *et al.*, 2003; Schiepers *et al.*, 2005). Moreover, cytokines including IL-1, IL-2 and IL-6 are also found to regulate neuronal functions (Blalock, 1994; Madden and Felten, 1995; Jiang *et al.*, 1998). IL-1 and IL-6 stimulate HPA axis in secretion of corticotrophin-releasing hormones (Blalock, 1994; McCann *et al.*, 1994), whereas IL-2 can act as a neuroregulatory cytokine in the CNS (Hanisch and Quirion, 1995; Jiang *et al.*, 1998). IL-2 can effect the growth and survival of neurites (Sarder *et al.*, 1993), proliferation and maturation of oligodendrocytes (Benveniste and Merrill, 1986), growth of microglia (Sawada *et al.*, 1995) and also has effect on behaviour and electrocorticogram spectrum (De Sarro *et al.*, 1990). It has also been shown that systemic immunisation in mice caused a decrease in neurotransmitter level in specific regions of the brain such as hypothalamus, hippocampus, locus coeruleus, supraoptic nucleus and paraventricular nucleus (Besedovsky *et al.*, 1983; Carlson *et al.*, 1987; Zalcman *et al.*, 1991) while causing an increase in the nucleus tractus solitarius (Carlson *et al.*, 1987). From these studies, it is evident that the CNS and the immune system communicate with each other via various mediators. Furthermore, there are also several studies showing a direct interaction between cells of the CNS and cells of the immune system (Aloisi *et al.*, 1998;

Greter *et al.*, 2005; Liu *et al.*, 2006). These immune cells are regularly patrolling the CNS and scanning for their cognate antigens in healthy as well as in diseased state like EAE (Hickey, 1991; Lampson *et al.*, 1994; Engelhardt and Ransohoff, 2005; Engelhardt, 2010). Details of their movement and functions are dealt with in the sections below.

1.4.3 Movement of lymphocytes in and out of the CNS

As discussed above, there is a continuous communication between the CNS and the immune system either via various neuromediators / immunomediators or direct cell to cell interactions. In direct cell to cell interactions, immune cells especially leukocytes migrate into the CNS. Three pathways have been proposed for leukocytes movement to and from the CNS: i) from blood to cerebro-spinal fluid (CSF) across the choroids plexus (the site of the blood-CSF barrier), ii) from blood to subarachnoid space at the pial surface of the brain and iii) from blood to parenchymal perivascular space (Ransohoff *et al.*, 2003). The first two pathways seem to be the most relevant under normal physiological conditions (Carrithers *et al.*, 2002) whereas extravasation by the third one occurs only during an inflammatory event (Hickey and Kimura, 1988; Hickey, 1991; Piccio *et al.*, 2002; Bartholomaus *et al.*, 2009). Normally, lymphocytes (predominantly T lymphocytes) migrate through the fenestrated endothelium of the choroid plexus stroma, interact with epithelial cells of the choroid plexus and enter the CSF at its site of formation (Seabrook *et al.*, 1998; Kivisäkk *et al.*, 2003; Ransohoff *et al.*, 2003). As described previously in section 1.3.1, activated T cells express specific chemokine receptors which enable them to interact with adhesion molecules expressed in the vascular endothelium (Engelhardt *et al.*, 1994; Jenkins *et al.*, 2001; Engelhardt, 2006). In this process, only activated lymphocytes can undergo this extravasation event, independent of antigen specificity (Hickey *et al.*, 1991) as these cells express chemokine receptors which help in this extravasation (Engelhardt and Wolburg/ 2004; Engelhardt, 2006; Axtell and Steinman, 2009; Reboldi *et al.*, 2009). This process of extravasation requires defined interactions between molecules expressed in lymphocytes and endothelial cells of the BBB. These discrete steps include interaction between selectins

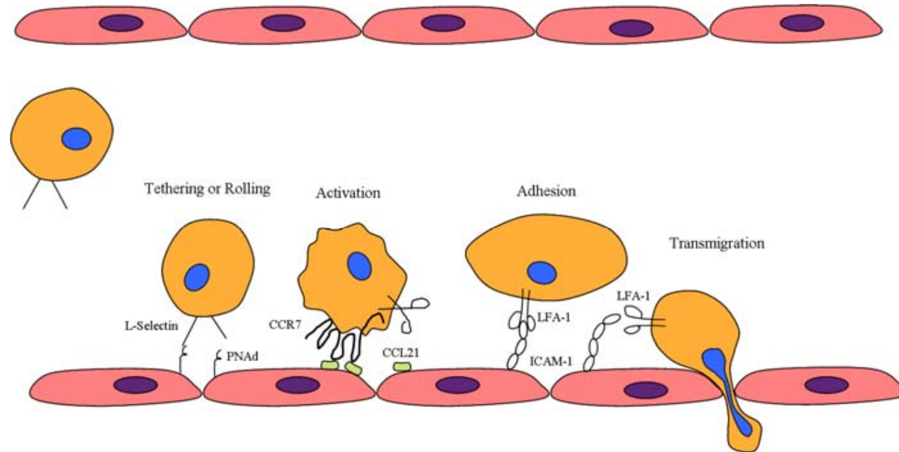
and their ligands, integrins and cell adhesion molecules (CAMs), chemokines and their receptors, matrix metalloproteinase (MMPs) and their tissue inhibitors (TIMPs).

The initial contact between a leukocyte and an endothelial cell is referred to as ''tethering'' and subsequent interactions are referred to as ''rolling'' occur in the peripheral high endothelial venules (Figure 1.6A). These initial events of leukocyte recruitment are primarily mediated by the interaction between P-selectin glycoprotein ligand-1 (PSGL-1; expressed by all lymphocytes) and selectins. However, the role of endothelial P-selectin in recruiting lymphocytes into the CNS is still controversial. Although, some studies have suggested that P-selectin has a crucial role in the early migration of lymphocytes into the non-inflamed brain (Carrithers *et al.*, 2002; Kerfoot and Kubes, 2002; Kivisäkk *et al.*, 2003), others have suggested that there is no role of P-selectin in migration of lymphocytes during inflammatory condition within CNS (Engelhardt *et al.*, 1997; Engelhardt *et al.*, 2005; Engelhardt, 2008) since P-selectin deficient mice also develop EAE indistinguishable from wild-type (Kerfoot *et al.*, 2006; Döring *et al.*, 2007). The "rolling" of lymphocytes followed by interaction of α4β1 molecule (α4-integrin) expressed in the membrane of lymphocytes with vascular cell adhesion molecule (VCAM)-1 expressed on the endothelial surface causes capture of these lymphocytes (Laschinger and Engelhardt, 2000; Vajkoczy *et al.*, 2001). This capturing process allows lymphocytes a sufficient time to interact with chemokine ligands (CCL19 and CCL21) expressed on the endothelial surface and leads to the activation as well as migration of lymphocytes into the brain (Alt *et al.*, 2002) and also causes them to adhere on the endothelial surface more firmly. In this stage lymphocyte function-associated antigen (LFA)-1 expressed on the membrane of lymphocytes, binds with intercellular adhesion molecule (ICAM)-1 or ICAM-2 expressed on the endothelial surface causing transendothelial migration of lymphocytes. The interaction between LFA-1 and ICAM-1 or ICAM-2 is considered to be crucial in the transendothelial migration of lymphocytes into the perivascular spaces of the CNS as it has been demonstrated that lack of ICAM-1 and ICAM-2 in mice fails to recruit lymphocytes into the CNS (Lyck *et al.*, 2003). Moreover, it is important to note that lymphocyte

recruitment in peripheral vascular endothelium is different than their recruitment into the perivascular area of the CNS. In the peripheral vascular endothelium, as explained above, there is a “tethering” and “rolling” of lymphocytes on the endothelial surface (Butcher *et al.*, 1999) which is absent in vascular endothelium of BBB (Figure 1.6B). In the vascular endothelium of BBB, there is immediate capture of the lymphocytes (predominantly T cells) followed by activation, adhesion and transmigration (Engelhardt, 2006).

Lymphocytes patrol the CNS regularly and scan for their cognate antigens in healthy as well as the inflamed CNS (Hickey, 1991; Hickey *et al.*, 1991; Engelhardt and Ransohoff, 2005). It has also been demonstrated that during a strong systemic immune response, there is an increase in lymphocytes (predominantly T cells) number in the CNS (Hickey and Kimura, 1987). Those T cells which recognise their cognate antigens/MHC complex initiate an immune reaction and reside in the CNS (Hickey *et al.*, 1991; Becher *et al.*, 2006; Odoardi *et al.*, 2007; Bartholomaus *et al.*, 2009) while those failing to recognise cognate antigens in the context of MHC molecules exit from the CNS (Hickey, 1999) suggesting that the entry of T cells into the CNS is dependent on their activation state rather than antigen specificity (Hickey *et al.*, 1991; Hirschberg *et al.*, 1998; Hickey, 1999).

(A)



(B)

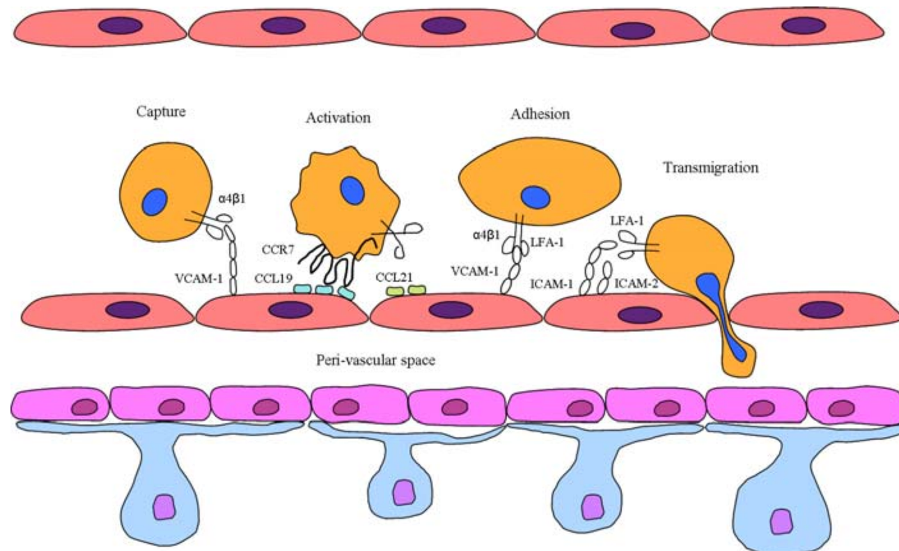


Figure 1.6: Schematic figure showing the multistep model of lymphcoytes movement in two different compartments.

(A) In the peripheral vascular endothelium, the extravasation of lymphocyte starts with tethering or rolling followed by activation, adhesion and transmigration. (B) At the endothelium of spinal cord, the extravasation of lymphocyte starts with direct capture without tethering or rolling and followed by activation, adhesion and migration into perivascular space. (Adapted from Butcher *et al.*, 1999; Engelhardt, 2006)

1.4.4 Interaction of lymphocytes and cells of the CNS

The interaction between lymphocytes and cells of the CNS and antigen presentation usually occurs in the perivascular spaces of the brain known as 'Virchow-Robin' spaces (Hickey *et al.*, 1991; Ransohoff *et al.*, 2003; Archambault *et al.*, 2005; Goverman, 2009). As described previously (see section 1.4.3), the initial entry of lymphocytes into the brain is suggested to be via the subarachnoid space, and/or the blood-CSF barrier, where selectins and adhesion molecules are expressed normally (Ransohoff *et al.*, 2003). Antigen presentation occurs between lymphocytes and microglial cells and also with astrocytes during neurodegenerative disease including MS (Bö *et al.*, 1994; Becher *et al.*, 2000; Zeinstra *et al.*, 2000; Neumann, 2001) but antigen presentation by astrocytes at the initial stage has been suggested to be unlikely as astrocytes did not show antigen presenting ability after IFN-γ in a culture model (Matsumoto *et al.*, 1992; Becher *et al.*, 2000). Several studies have confirmed that microglial cells can express MHC-II protein (Bö *et al.*, 1994; Neumann, 2001) and are effective antigen presenting cells (Becher *et al.*, 2000). Similarly, astrocytes also express MHC-II proteins and can act as antigen presenting cells (Zeinstra *et al.*, 2000; Neumann, 2001). Along with these cells, dendritic cells and macrophages, the professional antigen presenting cells, also help in antigen presentation to lymphocytes in the perivascular space (Becher *et al.*, 2000). Alternatively, it has also been shown that neurons interact with T cells via B7 ligand in the absence of MHC-II molecules as they do not express MHC-II molecules (Liu *et al.*, 2006).

Hence, from all these studies, it is evident that there is a definite communication between the CNS and the immune system. These two systems are interacting via various soluble factors as well as via direct cell to cell contact. Further, it is also apparent that these two systems are not working independently or are autonomous but working together to maintain proper physiological functions.

1.5 Role of lymphocytes in the CNS

It is now evident that lymphocytes are regularly patrolling the CNS and interacting with cells of the CNS (Hickey *et al.*, 1991; Engelhardt and Ransohoff, 2005). In normal CNS, these cells can exit from the CNS and scan for their cognate antigens/MHC molecules in the brain parenchyma. A study has suggested that T cells play a major role in the maintenance of normal mental activity and cognitive functions because mice devoid of mature T cells demonstrated a cognitive deficit and behavioural abnormalities which were restored by the adoptive transfer of T cells (Kipnis *et al.*, 2004). Moreover, there are various conditions of the brain, including most neurological disorders, in which lymphocytes infiltrate into the brain, reside in the CNS and initiate immune reactions (Hickey and Kimura, 1988; Becher *et al.*, 2000; Engelhardt and Ransohoff, 2005; Engelhardt, 2010). Several studies have reported that in neurological disorders especially, neurodegenerative disease, there is a massive recruitment of lymphocytes (predominantly T cells) into the CNS (Griffin *et al.*, 1987; Hickey, 1999; Alt *et al.*, 2002; Brochard *et al.*, 2009). However, the role of lymphocytes during neurodegeneration of brain is still controversial. A pathogenic role for lymphocytes has been suggested in neurodegenerative diseases including Parkinson's disease (Ip *et al.*, 2006; Appel *et al.*, 2009; Brochard *et al.*, 2009) while others argue their beneficial role in certain diseases (Cohen and Schwartz, 1999; Byram *et al.*, 2004; Beers *et al.*, 2008).

On the other hand, the role of B cells as immune cells is well understood. However their role in the CNS during inflammation is not clearly understood. The frequent detection of intrathecally produced immunoglobulins has suggested a role of B cells in the inflamed CNS (Pedemonte *et al.*, 2006). It has also been shown that B cells can seek their specific antigens in the CNS similar to T cells (Knopf *et al.*, 1998). When antigens are administered via indwelling cannula into the brain of pre-immunized mice adoptively transferred with antigen-specific B cells, B cells were found to accumulate at the site of antigen administration (Knopf *et al.*, 1998). On the other hand, in neuroinflammatory conditions of the CNS including MS and EAE, B cells are involved in the antigen

presentation (Corcione *et al.*, 2005) and differentiate into antibody secreting cells (Knopf *et al.*, 1998; Corcione *et al.*, 2005). It has been found that an increase in B cells number in MS can be correlated to the rapid progression of the disease (Cepok *et al.*, 2001). This study also suggested a possible role of B cells in the progression of MS, although their specific response is still unknown. There are few studies which have also shown the presence of ectopic lymphoid follicle-like structures with B cells, T cells and plasma cells in cerebral meninges of chronic neuroinflammatory condition of the CNS including MS and EAE model (Magliozzi *et al.*, 2004; Serafini *et al.*, 2004). Lymphoid follicle-like structures have been found in cerebral meninges of secondary progressive MS, but not in relapsing or primary progression MS and it has been suggested that this could be the crucial stage in initiating immune response and exacerbation of disease (Serafini *et al.*, 2004) since B cell responses are stimulated locally during autoimmune diseases (Magliozzi *et al.*, 2004). Therefore, under chronic inflammation, lymphoid follicle-like structures can develop at less immune-privileged site of meninges which support sustained survival of B cells during the CNS pathology (Magliozzi *et al.*, 2004). However, as I mentioned earlier, the role of B cells in the CNS during neuroinflammation is still poorly understood.

1.5.1 Recruitment of lymphocytes into the CNS in neurodegenerative diseases and inflammation

Neurodegenerative disease is a progressive loss of neurons from the CNS, associated with a deficit in the function of the affected region. Cell death during neurodegeneration can be either via apoptosis or necrosis or both (Martin *et al.*, 1998; DeLegge and Smoke 2008). Various conditions have been suggested for the neurodegeneration of the CNS including ageing (Collier *et al.*, 2011; Tollervey *et al.*, 2011), inflammation (Lucas *et al.*, 2006; Amor *et al.*, 2010), stress and trauma (Bramlett and Dietrich, 2004; Sugama, 2009) and genetic predisposition (Butterfield *et al.*, 2000; Elbaz *et al.*, 2007). Recent studies have shown a strong link between inflammation and neurodegeneration (Campbell 2004; Lucas *et al.*, 2006; DeLegge and Smoke, 2008) but the exact role for

inflammation in neurodegeneration is still elusive. It is not clear whether inflammation causes neuronal death in neurodegenerative diseases or whether the inflammatory infiltrate is simply a manifestation of the disease process. However, several possibilities have been proposed for the link between inflammation and neurodegeneration (Peterson and Fujinami 2007): 1) inflammation induces neurodegeneration, 2) neurodegeneration causes inflammation, 3) other factors cause either inflammation or neurodegeneration or both, 4) inflammation and neurodegeneration occur as a cycle which amplifies each others response and 5) inflammation can be neuroprotective in neurodegeneration. The key features of CNS inflammation are glial cell activation, local production of inflammatory mediators, expression of MHC and adhesion molecules, release of free-radicals and recruitment of immune cells (Lucas *et al.*, 2006).

During neurodegenerative diseases, either peripheral immune cells, such as T cells, initiate inflammation in the CNS (Hickey and Kimura, 1988; Neumann, 2001; Kivisäkk *et al.*, 2009) or CNS resident immune competent cells such as microglia as well as neurons, astrocytes and oligodendrocytes, release inflammatory mediators to recruit more peripheral immune cells including lymphocytes (Block and Hong 2005; Lucas *et al.*, 2006) leading to CNS inflammation. Most commonly, inflammation starts within subarachnoid space which disseminates to other regions of the brain (Lassmann and Wisniewski, 1978; Kivisäkk *et al.*, 2009). During inflammation of the CNS, endothelial cells of the BBB express various selectins and adhesion molecules (Kivisäkk *et al.*, 2002; Engelhardt and Wolburg, 2004; Reboldi *et al.*, 2009) that increase the migration of lymphocytes from the systemic circulation to the perivascular spaces of the brain. Further, activated lymphocytes also express various receptors including chemokines receptors, integrins and selectins that help to interact with their respective ligands expressed on the surface of endothelial cells during neuroinflammation (Piccio *et al.*, 2002; Ransohoff *et al.*, 2003; Goverman, 2009; Engelhardt, 2010). Activated lymphocytes and cells of the CNS including microglia, astrocytes, neurons and oligodendrocytes release various pro-inflammatory cytokines such as IL-1, TNF-α, IL-23, INF-γ and chemokines including various neurotrophic factors (Neumann, 2001;

Kerschensteiner *et al.*, 2009) which can contribute in the outcome of the CNS inflammation There are several neurodegenerative diseases including AD, MS, PD and stroke in which lymphocytes are actively involved and believed to be a key player in the initiation of CNS inflammation. Some examples of the most common neurodegenerative diseases are briefly explained in subsequent headings.

1.5.2 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that mostly affects patients in their later stage of life (Isik, 2010). Typical symptoms of AD are loss of cognitive functions including emotion, learning and memory processing skills leading to dementia (Mattson, 2004; Jalbert *et al.*, 2008). The pathological impression of AD can be characterised by the deposition of amyloid-beta (Aβ) protein plaques in the brain parenchyma and accumulation of tau proteins within neurons (Krause and Muller, 2010). These protein plaques are thought to interfere with synaptic transmission and neuron-neuron communication leading to neuronal death (Mattson, 2004; Ang *et al.*, 2010; Alzheimer's-Association, 2011). Further, high levels of tau proteins within neurons form tangles and block transportation of nutrients or other vital cellular factors throughout the cell (Ballatore *et al.*, 2007; Ang *et al.*, 2010; Alzheimer's-Association, 2011) which has been suggested to be one of the reasons for cell death in AD (Iqbal *et al.*, 2010; Alzheimer's-Association, 2011).

In AD, amyloid-beta plaques and tau proteins are considered to be crucial in the pathology. Recently, inflammation has been proposed to be one of the key players in AD (Akiyama *et al.*, 2000; Tahara *et al.*, 2006; Wyss-Coray, 2006). The inflammatory responses in AD can be characterised by the up-regulation of cytokines and chemokines along with activation of microglia (Akiyama *et al.*, 2000). The activated microglia clusters can be seen near amyloid-beta deposition site and these cells also express high levels of MHC-II, cytokines and chemokines (Griffin *et al.*, 1998; Streit *et al.*, 1999) contributing to disease progression (Banati *et al.*, 1993; Dickson *et al.*, 1993; Akiyama

et al., 2000). However, these microglial cells also involve in clearing of amyloid-beta and this function has been shown to be enhanced in the presence of TGF-β (Wyss-Coray *et al.*, 2001; Wyss-Coray, 2006). In addition, reactive astrocytes are also clustered at the site of amyloid-beta deposition but high levels of these cells also accumulate at neuritic plaques (Mrak *et al.*, 1996). These reactive astrocytes are capable of expressing various cytokines (Del Bo *et al.*, 1995; Hampel *et al.*, 2005), growth factors (Flanders *et al.*, 1998), adhesion molecules (Akiyama *et al.*, 1993) and prostaglandins (Hirst *et al.*, 1999). Furthermore, astrocytes are suggested to be involved in inhibition of microglial ability to clear amyloid beta (DeWitt *et al.*, 1998). The analysis of brain autopsy has also revealed that there is a significant increase in inflammatory markers (Lue *et al.*, 1996) as well as increase in complement activation and lysis of neurites (Webster *et al.*, 1997) in AD subjects when compared to non-demented subjects, which strongly suggests that inflammation has a role in AD. With these findings, it can be suggested that this inflammatory responses might contribute in recruiting lymphocytes from the systemic circulation into the brain. Further, T cells have been detected in the brain of AD patients (Togo *et al.*, 2002). Recent studies have also demonstrated the up-regulation of T cells in the CNS of AD when compared to healthy controls (Saresella *et al.*, 2010; Saresella *et al.*, 2011). These studies have revealed an increased activity of various subsets of T cells such as Th-17 and Th-9 in AD and cytokines including IL-9, IL-21 and IL-23 released from these T cells are also increased in AD which have been suggested to be one of the factors in AD-associated neuroinflammation (Saresella *et al.*, 2011). The beneficial effect of T cells has been demonstrated in AD however, these T cells lose their protective effect in severe condition of AD (Saresella *et al.*, 2010). With these findings, it is evident that lymphocytes are present in the brain of AD and may have an important role in AD. However, the role of lymphocytes in AD is still poorly understood and a better understanding of this phenomenon could help in search of novel drug targets. Therefore, further studies are required to explore the exact role of lymphocytes in AD.

1.5.3 Multiple sclerosis

Multiple sclerosis (MS) is a chronic neuroinflammatory disease of the CNS characterised by demyelination, axonal damage and autoimmunity (Bö *et al.*, 2006; McFarland and Martin, 2007; van Horssen *et al.*, 2011) affecting people mostly between of 20 and 40 (Tienari, 1994). It affects both white and gray matter of the CNS and scattered focal demyelinated lesions can be seen through out the white matter of the CNS (Hafler, 2004; Bö *et al.*, 2006). There is an episodic exacerbation followed by remission during the course of the disease and this relapsing-remitting course is suggested to be immune-mediated (Noseworthy *et al.*, 2000; van Horssen *et al.*, 2011) which is characterised by activation of microglia and infiltration of peripheral immune cells into the CNS. This disease process leads to secondary MS having marked degeneration of neurons and axons along with massive cortical demyelination (Bö *et al.*, 2003; Bö *et al.*, 2006; Frohman *et al.*, 2006; van Horssen *et al.*, 2011).

Genetic predisposition (The_International_Multiple_Sclerosis_Genetics_Consortium and The_Wellcome_Trust_Case_Control_Consortium_2, 2011), environmental factors (Munger *et al.*, 2006; Handel *et al.*, 2011) and viral/microbial infections (Sundström *et al.*, 2004; Thacker *et al.*, 2006) have been proposed as risk factors in MS but how these factors contribute to the aetiology of the disease is still under investigation. However, it is now well accepted that MS is a T cell mediated autoimmune disease (Noseworthy *et al.*, 2000; O'Connor *et al.*, 2001; McFarland and Martin 2007; Peterson and Fujinami, 2007). The concept of MS as an autoimmune disease arises due to its similarities in clinical symptoms with EAE, an animal model for this disease (Comabella and Khoury, 2011). EAE is induced in the animal by immunising with myelin-derived protein such as myelin-basic protein, proteolipid protein or myelin oligodendrocyte glycoprotein and the disease is mainly initiated by myelin-specific autoreactive T cells. Autoreactive T cells have been identified in MS patients, as well as being present in healthy people (Diaz-Villoslada *et al.*, 1999). However, these T cells are more activated and have a memory phenotype in MS when compared to healthy subjects (Lovett-Racke *et al.*, 1998; Frohman *et al.*, 2006). These activated T cells express various chemokines, cytokines

and adhesion molecules which help them to interact with the BBB and migrate into the CNS to initiate immune response against myelin-derived proteins. Although, $CD4^+$ T cells are thought to be initiators of EAE, analysis of MS lesioned brain tissues has shown predominance of $CD8^+$ T cells (Babbe *et al.*, 2000). $CD4^+$ T cells can be either neuroprotective or pathogenic depending on the types of cytokines or neurotrophic factors they release (Peterson and Fujinami ,2007). As described above, different subsets of $CD4^+$ T cells release their functional cytokines: Th1 cells release inflammatory mediators including IFN-γ and TNF-α whereas Th-2 cells release anti-inflammatory mediators like IL-4 (Mosmann *et al.*, 1986). IFN-γ and TNF-α have shown a contrast in response in EAE and MS since IFN-γ exhibits neuroprotection in rodent models of EAE (Peterson and Fujinami, 2007) whereas it exacerbates the disease in MS (Panitch *et al.*, 1987). Similarly, blocking TNF-α function in EAE is neuroprotective but not in MS (Lassmann and Ransohoff, 2004). This finding therefore suggests that caution should be taken while comparing data from the EAE model with MS. The major concept of immunopathogenesis of MS has been connected to the balance between Th-1 and Th-2 functions. However, there are other subsets of $CD4^+$ T cells such as Th-17 and T_{reg} cells along with $CD8^+$ T cells cannot be excluded. Th-17 cells have been found to express more activation markers, co-stimulatory and adhesion molecules than Th-1 cells (Brucklacher-Waldert *et al.*, 2009) suggesting they are more pathogenic. Further, the pathogenic T cells suppressor capacity of T_{reg} cells is found to be attenuated in MS (Viglietta *et al.*, 2004). Moreover, $CD8^+$ T cells have been directly linked to the demyelination of axons in MS (Neumann *et al.*, 2002) and are pathogenic in the immune-mediated demyelination of axons (Lassmann and Ransohoff, 2004). Nevertheless, there is no exact mechanism how these cells are contributing to the disease process or neuroprotection in MS and further research is required to understand MS pathology.

1.5.4 Parkinson's disease

Parkinson's disease (PD) is an age-related chronic neurodegenerative disease clinically characterised by tremor, rigidity, bradykinesia, postural instability, dementia and autonomic dysfunction (Forno, 1996; Olanow and Tatton, 1999; Ang *et al.*, 2010) while pathologically by loss of dopaminergic neurons in the substantia niagra and the presence of Lewy's bodies which are aggregated proteins such as α-synuclein (Irvine *et al.*, 2008; Ang *et al.*, 2010). The accumulation and misfolding of α-synuclein induce toxicity leading to the loss of dopaminergic neurons (Obeso *et al.*, 2010). This results in a reduction of dopamine production causing gait and movement impairment because dopamine is required for a normal motor function of the brain (Forno, 1996). Further, proteosomal and lysosomal system dysfunction and reduction in mitochondrial activity due to genetic mutations are also proposed to be causative factors in neuronal death during PD (Obeso *et al.*, 2010).

Various risk factors including environmental (Koller *et al.*, 1990; Tanner and Langston 1990), genetics (Golbe, 1990; Olanow and Tatton, 1999) and age (Collier *et al.*, 2011) have been related to pathogenesis of PD. Furthermore, there are several studies suggesting the relation between inflammation and pathology of PD (Hunot and Hirsch, 2003; Imamura *et al.*, 2003; Lee *et al.*, 2009; Obeso *et al.*, 2010). However, it is still not clear whether inflammation observed in PD can be considered as classical inflammation or not (Hunot and Hirsch, 2003). Therefore, the term 'neuroinflammation' has been coined in the pathology of PD. The upregulation of MHC expression is one of the first signs of neuroinflammation in PD (McGeer *et al.*, 1988; Hunot and Hirsch, 2003) with an increase in MHC-II expressed microglia in the substantia niagra. Similar upregulation of MHC molecules has been reported in 1-methyl-4-phenyl-1,2,3,6-tetrahydropuridine (MPTP)-intoxicated animals, an animal model for PD and is also associated with the infiltration of lymphocytes in substantia niagra (Kurkowska-Jastrzebska *et al.*, 1999). The other hallmarks for neuroinflammation are the presence of reactive astrocytes, activated microglia, increase in cytokines, chemokines, prostaglandins, and reactive oxygen and nitrogen species (Ransohoff and Perry, 2009). Microglial activation has

been related to accumulation of α-synuclein protein and proteosomal and lysosomal system dysfunction (Lee *et al.*, 2009) and these activated microglial cells have been reported to induce cell death in dopaminergic neurons (Le *et al.*, 2001). Furthermore, neurons over-expressing α-synuclein protein have demonstrated early activation of microglia and release of various inflammatory mediators such as IL-1, IL-6 and TNF-α and inflammation-related enzymes including cylcoxygenase-2 (COX-2) and inducible nitric oxide synthase (Knott *et al.*, 2000; Ciesielska *et al.*, 2003; McGeer *et al.*, 2005; Griffin *et al.*, 2006). In addition, pro-inflammatory factors including IL-1, TNF-α, reactive oxygen/nitric oxide species and prostaglandins released from the activated microglia induced by α-synuclein protein can enhance oxidative stress and trigger cell-death pathways (Liberatore *et al.*, 1999; McGeer and McGeer, 2004; Klegeris *et al.*, 2008).These locally released inflammatory mediators also induce expression of cellular adhesion molecules in the endothelium of the BBB which help in the subsequent recruitment of immune cells from the periphery into the inflammatory site (Engelhardt, 2008). Several studies have shown the infiltration of lymphocytes into the CNS in PD (Brochard *et al.*, 2009; Huang *et al.*, 2009; Rezai-Zadeh *et al.*, 2009; Chung *et al.*, 2010). These immune cells including leukocytes, macrophages, B cells and T cells, predominantly, $CD4^+$ T cells and $CD8^+$ T cells are recruited in the substantia niagra region of the brain in PD (Brochard *et al.*, 2009; Chung *et al.*, 2010) with $CD4^+$ T cells being suggested to be cytotoxic in PD (Brochard *et al.*, 2009). However, the other subsets of T cells such as T_{reg} cells are also present in the substantia niagra of PD and have the ability to suppress the cytotoxicity of effector T cells like Th-17 cells (Reynolds *et al.*, 2007; Reynolds *et al.*, 2009; Reynolds *et al.*, 2010). These T_{reg} cells can also induce apoptosis in activated microglia and reduce α-synuclein protein induced neurotoxicity (Reynolds *et al.*, 2007; Reynolds *et al.*, 2009). Although various studies have argued on either the pathogenic or the neuroprotective role of lymphocytes, the precise role of lymphocytes in PD is still elusive. Hence, it is evident that lymphocytes play a key role in PD but, their role in relation to PD pathology still needs to be explored extensively.

1.5.5 Stroke

The World Health Organisation (WHO) has defined stroke as the clinical syndrome of rapid onset of focal or global cerebral deficit, lasting more than 24 hours or leading to death with no apparent cause other than a vascular one (Warlow *et al.*, 2003). Various risk factors including smoking, diabetes, hypertension, atrial fibrillation and transient ischaemic attack have been identified for stroke (Donnan *et al.*, 2008) which can lead to either haemorrhagic or ischaemic stroke. Haemorrhagic stroke is due to hypertensive arteriosclerosis and amyloid angiopathy (Auer and Sutherland, 2005) whereas, the major cause of focal or ischaemic stroke is due to the occlusion of major arteries of the brain and accounts for 80% of all strokes (Flynn *et al.*, 2008). The occlusion in the artery within the CNS leads to a reduction of blood flow leading to insufficient oxygen and glucose supply to the brain. Usually, this occlusion develops due to thrombosis in situ (such as atherothrombosis of large cervical or intracranial arteries), or embolism from heart or blockage of small penetrating arteries due to arteritis or haematological disorders (Hossmann, 1994). In stroke, a series of neurochemical processes occurs which is termed an ischaemic cascade. During ischaemic cascades, various events take place including cellular metabolic failure due to hypoperfusion, oxidative stress, excitoxicity, damage of BBB, microvascular injury, activation of haemostatic system and inflammation (Brouns and De Deyn, 2009). These series of events result in non-selective cell death in the CNS including neurons, astrocytes, microglia, oligodendrocytes and endothelial cells (Sims and Muyderman, 2010).

Apart from various risk factors including smoking, diabetes and hypertension, there are several studies showing a relation between inflammatory status and the risk of stroke (McColl *et al.*, 2007; Wang *et al.*, 2007; McColl *et al.*, 2009; Denes *et al.*, 2010) which also has an effect on the outcome of stroke (Denes *et al.*, 2010). The inflammatory response arises from the series of ischaemic cascades. The hypoperfusion occurs during this cascade causes a failure of energy-dependent ion pump leading to activation of calcium channels (Katsura *et al.*, 1994) and release of excitatory neurotransmitters including glutamate into extracellular spaces (Nicholls and Attwell, 1990; Martin *et al.*,

1994). The release of excess excitatory neurotransmitters causes neuronal death which is termed as excitotoxicity (Meldrum *et al.*, 1985; Rothman and Olney, 1986; Choi, 1988; Brouns and De Deyn, 2009). On the other hand, a disturbance in the scavenging of free reactive radicals causes oxidative stress (Coyle and Puttfarcken, 1993; Langley and Ratan, 2004; Brouns and De Deyn, 2009). There is significant evidence illustrating the cytotoxic effects of free reactive oxygen and nitrogen species (Nelson *et al.*, 1992; Love, 1999; Chan, 2001). This oxidative stress leads to glial cells activation followed by release of various inflammatory mediators including cytokines, chemokines and reactive free radicals (Dong and Benveniste, 2001; Wang *et al.*, 2007), as well as expression of MHC I and II and co-stimulatory molecules (Morioka *et al.*, 1993). The level of IL-1 has been reported to be elevated after experimental stroke (Minami *et al.*, 1992; Liu *et al.*, 1993; Denes *et al.*, 2010) and has been a target for therapy in stroke in reducing inflammation-related damage (Brough *et al.*, 2011).

Non-specific activation of peripheral T cells has been reported in stroke patients compared with healthy subjects (Yan *et al.*, 2009), and the number of T_{reg} cells is increased in stroke patients, similar to that described in animal models (Offner *et al.*, 2006; Yan *et al.*, 2009). During inflammation, endothelial cells of the BBB express various adhesion molecules including ICAM-1, ICAM-2 and VCAM-2 and selectins that help lymphocytes migration from the periphery to the CNS (Engelhardt *et al.*, 1994; Stoll *et al.*, 1998; Blann *et al.*, 2002; Tanne *et al.*, 2002). These lymphocytes express chemokine receptors, α4β1 integrins and LFA-1 helping them in capturing, activation and transendothelial migration across the BBB (Engelhardt, 2008, 2010). Several studies have shown infiltration of lymphocytes into the CNS following stroke (Schroeter *et al.*, 1994; Gee *et al.*, 2007; Ortolano *et al.*, 2010; Fumagalli *et al.*, 2011). Real time *in vivo* imaging of the mouse brain after experimental stroke revealed a massive number of T cells infiltration into the stroke brain in comparison to sham (Fumagalli *et al.*, 2011). This recent study has reported two distinct populations of T cells in experimental stroke, the fast migrating T cells and slow migrating T cells (Fumagalli *et al.*, 2011) but, the definitive role of these two populations in stroke is still under investigation.

Furthermore, various studies have shown the neurotoxic effect in the stroke (Hurn *et al.*, 2007; Liesz *et al.*, 2011) while at the same time others have argued on their neuroprotective role in the stroke (Frenkel *et al.*, 2005; Planas and Chamorro, 2009; Ren *et al.*, 2011). Taken together, to date there has been little agreement on the precise role of lymphocytes in the stroke and a better understanding of their contribution to stroke is still required.

1.6 Role of lymphocytes in neurodegenerative diseases: pathogenic or neuroprotective?

Infiltration of lymphocytes into the CNS during neurodegenerative diseases is well established (McFarland and Martin, 2007; Peterson and Fujinami, 2007; Rezai-Zadeh *et al.*, 2009; Chung *et al.*, 2010; Fumagalli *et al.*, 2011; Saresella *et al.*, 2011) and the molecular mechanisms underlying their recruitment into the CNS has also been well documented (Engelhardt, 2006; Engelhardt, 2008, 2010). However, the controversy of scientific evidence for the role of lymphocytes during neurodegeneration has raged unabated for more than a half century.

It is now evident that after infiltration into the CNS and recognition of cognate antigen/MHC, peripherally activated lymphocytes (see section 1.3.1) can initiate inflammatory response in the CNS (Engelhardt and Ransohoff, 2005; Engelhardt, 2010) which can be either neuroprotective or neurotoxic. The pathogenic role of T cells has been demonstrated in neurodegenerative diseases causing neuronal death (Fee *et al.*, 2003; Appel, 2009; Brochard *et al.*, 2009; Huang *et al.*, 2009). The extent of the CNS injury during neurodegeneration has been correlated with the increase in T cells infiltration into the CNS (Popovich *et al.*, 1997) suggesting the greater the infiltration, the greater the neuronal injury. It has been suggested that these infiltrating T cells can also mediate cell death and demyelination in neurodegenerative diseases, affecting other effector cells including mircoglia and/or macrophages (Popovich *et al.*, 1996). The adoptive transfer of T cells from spinal cord injury model mice and EAE-induced mice

to healthy recipients develops paralytic disease (Popovich *et al.*, 1996), which further supports the pathogenic role of T cells. It has been shown that during neurodegeneration and brain injury, both T cells and B cells are activated which is referred to as auto-reactive T cells or B cells (Wang *et al.*, 1992; Olsson *et al.*, 1993). The number of auto-reactive T cells is increased in neurodegeneration and CNS trauma (Wang *et al.*, 1992; Olsson *et al.*, 1993; Popovich *et al.*, 1997; Kil *et al.*, 1999) and they predominantly release IFN-γ and TNF-α (Wang *et al.*, 1992; Kil *et al.*, 1999). Moreover, these cytokines released by these auto-reactive cells can exacerbate ischaemia and excitotoxicity in the brain during neurodegeneration (Viviani *et al.*, 2004). Studies have also demonstrated that TNF-α induces cell death via apoptotic pathways (Hsu *et al.*, 1996; Mogi *et al.*, 2000) and its concentration was also found to be elevated during neurodegenerative disease including PD (Mogi *et al.*, 2000). In addition, activated $CD4^+$ T cells express Fas-ligand (FasL), which has been reported to induce cell death via apoptosis in neurodegenerative diseases including EAE (Nagata and Suda, 1995; Dittel 2000). These Fas and FasL are type I and II transmembrane receptors belonging to TNF/nerve growth factor and TNF families' protein respectively (Nagata and Golstein, 1995). The infiltrating $CD4^+$ T cells in PD induce apoptosis of dopaminergic neurons via FasL-Fas interaction (Brochard *et al.*, 2009), mediating FasL-mediated activation of microglia and neurodegeneration. The up-regulation of Fas and their ligands have been demonstrated in the CNS during neurodegenerative disease such as EAE (Sabelko-Downes *et al.*, 1999) leading to the apoptotic cell death. In addition, $CD8^+$ T cells or cytotoxic T lymphocytes (CTL) are proposed to be involved in direct killing of neurons in a MHC-I dependent manner (Medana *et al.*, 2000; Medana *et al.*, 2001). The induction of MHC-I expression in neurons via IFN-γ has been documented (Neumann *et al.*, 1995; Medana *et al.*, 2000) and it has also been reported that the cytotoxicity of CTL in these neurons is mediated via either FasL-mediated neuronal apoptosis (Medana *et al.*, 2000) or perforin-dependent lysis of neurons (Rensing-Ehl *et al.*, 1996). Moreover, both $CD4^+$ T cells and $CD8^+$ T cells have been reported to be equally neurotoxic and mediated via direct cell contact mechanism involving FasL, LFA-1 and CD40 (Giuliani *et al.*, 2003).

Despite the proposed role of T cells in neurodegeneration, there is growing evidence for a beneficial or neuroprotective role of lymphocytes in neurodegenerative diseases (Moalem *et al.*, 1999; Moalem *et al.*, 2000; Wolf *et al.*, 2002; Schwartz, 2005; Beers *et al.*, 2008). Adoptive transfer of auto-reactive T cells from EAE-induced mice to healthy recipient induces pathology (Popovich *et al.*, 1996). However, when these cells are transferred to the mice with partial optic nerve crush, a model for secondary neurodegeneration, they were found to be beneficial (Moalem *et al.*, 1999). Nerve cells from the mice which received auto-reactive T cells were found to survive well and were resistant to secondary neurodegeneration. Further analysis also revealed that only T cells specific to MBP were able to protect retinal ganglion cells from secondary damage and not T cells specific for non-self antigens, such as ovalbumin and heat-shock proteins. They further have suggested that only CNS-specific T cells are activated at the injured site to exert the neuroprotective response while T cells specific to non-self antigens fail to activate because of inadequate antigen recognition (Moalem *et al.*, 1999). In addition, after experimental axotomy of facial nerves, facial motor neurons are found to be severely impaired in severe combined immunodeficient (SCID) mice which lack T cells and B cells (Serpe *et al.*, 1999; Serpe *et al.*, 2003) but are restored up to wild-type controls after adoptive transfer of wild-type splenocytes containing T and B cells. Similarly, intraperitoneally injection of auto-reactive MBP-specific T cells in rat with an experimentally crushed spinal cord show early recovery with greater locomotor function as compared to controls (Hauben *et al.*, 2000; Hauben *et al.*, 2000). Moreover, an *in vitro* study in murine entorhinal-hippocampal brain slices shows down-regulation of the Th-1 cells induced inflammatory marker, ICAM-1 in microglia (Gimsa *et al.*, 2001) while another study demonstrated the neuroprotective role of both Th-1 and Th-2 cells (Wolf *et al.*, 2002).

It is now well understood that upon activation by their cognate antigen/MHC-II, $CD4^+$ T cells in the presence of appropriate mediators also differentiate into T_{reg} cells (Nakamura *et al.*, 2001; Chen *et al.*, 2003; Bettelli *et al.*, 2006) and several studies have

demonstrated the immunosuppressive role of T_{reg} during neuroinflammation (Liu *et al.*, 2006; Offner *et al.*, 2006; Reynolds *et al.*, 2007; Liu *et al.*, 2009; Reynolds *et al.*, 2010). T_{reg} cells have an immunomodulatory role in human immunodeficiency virus (HIV)-1-induced neurodegeneration leading to neuroprotection by suppressing microglial activation and secreting neurotrophic factors (Liu *et al.*, 2009). T_{reg} cells are able to salvage neurons by suppressing the inflammatory response mediated by Th-17 cells (Reynolds *et al.*, 2010). It has also been demonstrated that interaction between neurons and pathogenic T cells in EAE-induced mice cause conversion of pathogenic T cells into T_{reg} cells which are able to suppress neurodegeneration induced by pathogenic T cells (Liu *et al.*, 2006). The conversion of pathogenic T cells into neuroprotective T_{reg} cells has been suggested to be induced by the interaction of $CD4^+$ T cells and neurons via B7-CD28 and TGF-β receptor signalling pathways (Liu *et al.*, 2006). In addition, IL-10 producing $CD4^+$ T cells are found to be neuroprotective in stroke (Frenkel *et al.*, 2005) and it has been demonstrated that IL-10 and TGF-β can modulate immune processes by inhibiting Th-1-induced inflammatory responses as well as general inflammation leading to neuroprotection (Weiner, 2001; Frenkel *et al.*, 2005; Xin *et al.*, 2011). These auto-reactive T cells are able to produce various neurotrophic factors including brain-derived neurotrophic factors (BDNF), neurotrophin-3 (NT-3) and glial-cell derived neurotrophic factors which can rescue neurons from neurodegeneration (Hammarberg *et al.*, 2000; Moalem *et al.*, 2000). It has also been suggested that T cells can instruct microglia to remove neurotoxic extracellular glutamate resulting in neuroprotection (Shaked *et al.*, 2005).

Investigating the role of B cells in EAE-induced mice reveals that B cell deficient mice are more susceptible to EAE induction developing severe pathology with delayed recovery and early demyelination in compare to their controls (Wolf *et al.*, 1996; Hjelmström *et al.*, 1998). Likewise, a decrease or absence of B cells in EAE-induced mice correlates with an increase in severity of disease as well as influx of more pathogenic T cells into the CNS (Matsushita *et al.*, 2006; Matsushita *et al.*, 2008). In addition, IL-10 secreted by B cells is reported to be neuroprotective in EAE-induced

mice since EAE-induced mice having IL-10 deficit B cells failed to recover and manifested persistent inflammatory responses (Fillatreau *et al.*, 2002). Hence, IL-10 specific B cells are suggested to be playing a crucial role in the recovery and progression of the disease (Matsushita *et al.*, 2008). It has also been demonstrated that B cells are able to limit the spreading and severity of disease in EAE-induced mice (Lyons *et al.*, 2008). Furthermore, there is evidence showing that B cells are able to release various neurotrophic factors including BDNF, NT-3 and neurotrophic growth factor (Kerschensteiner *et al.*, 1999; Edling *et al.*, 2004; Kala *et al.*, 2010) which have a possible role in contributing to neuroprotection in neurodegenerative diseases.

Thus, it is clear that the notion of the brain as an absolute immune-privileged site is no longer appropriate, rather it can be considered as a site of active immune-surveillance. Recent evidence suggests the existence of bidirectional communication between these two systems and can influence each other via various mediators. In addition to this bi-directional communication, several studies have reported that lymphocytes are regularly patrolling the normal CNS in low number which can be increased upon recognition of cognate antigen and initiation of inflammation at the site. Whilst many attempts have been made to describe either the pathogenic or the neuroprotective role of lymphocytes, there has been little discussion about precise function(s) and mechanism(s) of these responses during neurodegeneration.

1.7 Why is it important to study?

The global burden of neurodegenerative diseases is huge and accounts for at least 15% of the global burden of diseases (Cruz *et al.*, 2006). Although cases of neurodegenerative disease are increasing every year, reliable therapeutics are still being sought and a complete understanding of the underlying biology is lacking. Moreover, as I have described previously, lymphocytes have been implicated in various neurodegenerative diseases and have been suggested to be one of the key player in these conditions. However, their role in neurodegenerative diseases is still poorly understood.

Therefore, by defining the role and underlying mechanism(s) of lymphocytes and other related immune cells in neurodegenerative diseases, I believe that it could be a milestone for the better understanding of the disease pathology and its therapy.

1.8 Working hypothesis: aim and objectives

As highlighted above, despite significant interest in the role of lymphocytes in CNS disorders, their role is still debatable. In this thesis, I have investigated the role of lymphocytes in the CNS by testing the hypothesis, "Lymphocytes play a crucial role in the CNS during neurodegenerative diseases." To test this hypothesis, I have used organotypic cortico-hippocampal cultures as an *in vitro* neurodegenerative model by addressing the following specific questions.

- What role do lymphocytes play in the CNS during normal physiological conditions and in diseased states?
- Do neuronal and non-neuronal components affect the outcome of lymphocytes in neurodegenerative diseases?
- What are the underlying cellular mechanism(s) exhibited by lymphocytes in neuronal and non-neuronal cells?
- Do lymphocytes play different roles depending on the disease model?

2 MATERIALS AND METHODS

2.1 Materials

Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 555 anti-mouse IgG, Alexa Fluor 633 anti-rat IgG, B-27, foetal calf serum (FCS), L-Glutamine, Hank's balanced salt solution (HBSS), horse serum (heat inactivated; Org. New Zealand), minimum essential medium (MEM), mouse anti-phospho extracellular signal-regulated kinases (ERK), Neurobasal-A, propidium iodide (PI), rabbit anti-phospho c-Jun N-terminal kinases (JNK) and rabbit anti-phospho p38 mitogen-activated protein kinases (MAPK) were obtained from Invitrogen (Paisley, UK). Mouse serum Diagnostics Scotland, Edinburgh (UK) and RPMI-1640 was bought from PAA laboratories (Somerset, UK). Biotinylated-anti-mouse CD3e (Clone: 145-2C11), purified NA/LE hamster anti-mouse CD3e (Clone: 145-2C11), purified NA/LE hamster anti-mouse CD28 (Clone: 37.51), hamster anti-mouse CD69 FITC or PE (Clone: H1.2F3), hamster anti-mouse IgG_1 FITC or PE or APC (Clone: G235-2356), rat anti-mouse CD4 FITC (Clone: H129.19) or APC (Clone: RM4-5), rat anti-mouse CD8 PE or PerCP (Clone: 53-6.7), rat anti-mouse CD62L FITC (Clone: MEL-14), rat anti-mouse IgG_{2a} APC (Clone: R35-95), rat anti-mouse IgG_{2a} PerCP (Clone: R35-95), rat anti-mouse IgG_{2a} FITC (Clone: R35-95) and streptavidin horseradish peroxidase (HRP) FITC or APC were obtained from BD Pharmingen (Oxford, UK). Pan T cell Isolation Kit was bought from Miltenyi Biotec (Surrey, UK). (2S,3S,4S)-3-(carboxymethyl)-4-(prop-1-en-2-yl)pyrrolidine-2-carboxylic acid (kainic acid), 4-[4-(4-Fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1H-imidazol-5-yl]pyridine hydrochloride (SB20203580), 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY294002), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and DL-(-)-2-amino-5-phosphonopentanoic acid (DL-AP5) were obtained from Ascent Scientific (Bristol, UK). D-Glucose, ethylene-di-amine tetra-chloro-acetate (EDTA), sodium azide (NaN_2), sodium fluoroacetate (FAc), paraformaldehyde (PFA), sodium chloride, anti-microtubule-associated protein (MAP)-2 (Clone: HM-2), bovine serum albumin (BSA), phosphate buffer tablets, methanol,

ethanol and Triton X-100 solution were obtained from Sigma-Aldrich (Poole, UK). Calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), potassium chloride (KCl), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$) and sucrose were obtained from VWR (Lutterworth, UK). Rabbit anti-total p38 MAPK, rabbit anti-total ERK and rabbit anti-total JNK were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Vectashield with DAPI was from Vector Laboratories (Peterborough, UK). Anti-mouse CD11b (Clone: 5C6) was from AbD SeroTec (Kidlington, UK). Anti-green fluorescent protein or anti-GFP was bought from Clontech (Mountain View, USA). Anti-mouse horseradish peroxidase-conjugated antibody and anti-rabbit horseradish peroxidise-conjugated antibody were obtained from Jackson Immuno Research Laboratories (Newmarket, UK). 6-well tissue culture plates were obtained from Becton Dickinson (Bedford, USA). Millex syringe-driven filter unit (0.22 µm) was obtained from Millipore Ireland Ltd. (Carrigtwohill, Ireland).

2.2 Methods

2.2.1 Preparation of organotypic cortico-hippocampal slice culture

2.2.1.1 Preparation of media and culture plates

Two types of media were prepared for organotypic cortico-hippocampal slice culture (OSC): preparation media and culture media. The preparation media contains 25 mM D-glucose, 50% Minimum essential media, 25% Hank's balanced salt solution, 25% heat-inactivated horse serum and 1mM L-Glutamine. All the constituents were mixed in a sterile bottle and were filtered through a syringe filter into a sterile tube.

The culture media is a serum-free media which contains 97% Neurobasal-A, 2% B-27, a serum-free supplement and 1% L-Glutamine (200 mM stock concentration). All the constituents were mixed in a sterile tube and filtered through a syringe filter into sterile tube.

OSCs were cultured in preparation media on the first day following dissection and preparation of OSCs. The preparation media was replaced by culture media from second day *in vitro* (DIV) for the rest of the culture period. All the procedures were carried out under aseptic conditions and all media preparations were antibiotic free.

2.2.1.2 Organotypic cortico-hippocampal slice culture preparation

C57Bl/6 mouse pups (4-7 days old) were obtained from the Biological Procedure Unit (BPU) of Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS), University of Strathclyde. This age of mouse pups was found to be more suitable for culture as slice culture from older animals did not survive well. It is a limitation of this culture system as for a model of neurodegenerative diseases as these conditions occur mostly at the later stage of life. Each pup was killed by cervical dislocation and decapitated according to Schedule 1 of the UK Home office guidelines. The head was placed into the ice-cold 70% ethanol and transferred to 70% ethanol sterilised tissue paper. The scalp bones were removed and the brain was exposed. The cerebellum was cut-off and the brain was transferred into a sterile petri-dish containing ice-cold preparation media. The brain was bisected and each hemisphere glued down on a sterile specimen bath. These brain halves were submerged in the ice-cold preparation media and 300 µm thick parasaggital slices were cut using a vibratome (Intracel, UK). At least 6 slices, 3 from each hemisphere, were obtained and transferred to a fresh petri-dish containing ice-cold preparation media.

The hippocampus and cortex were dissected out from the parasaggital slice (Figure 2.1) using a scalpel under a binocular microscope and collected in sterile petri-dish containing ice-cold preparation media. These slices were transferred to 6-well culture plates containing Millicell membrane inserts (0.4 µm; 30 mm diameter) which had been prepared previously. Slices were cultured in 6-well culture plates with a membrane insert in each well. The culture plate, inserts and media were incubated for at least 1

hour at 37 °C, 5% CO_2 prior to transfer of slices onto the membrane inserts to equilibrate pH and temperature. The insert was maintained in media (1 ml) underneath and the plates incubated at 37 °C, 5% CO_2. Slices were incubated in preparation media on the first day of culture which was then replaced by culture media after 24 hours. The culture media was changed 3 times per week and OSCs were cultured for 13-15 day *in vitro* (DIV) prior to use for experiments.

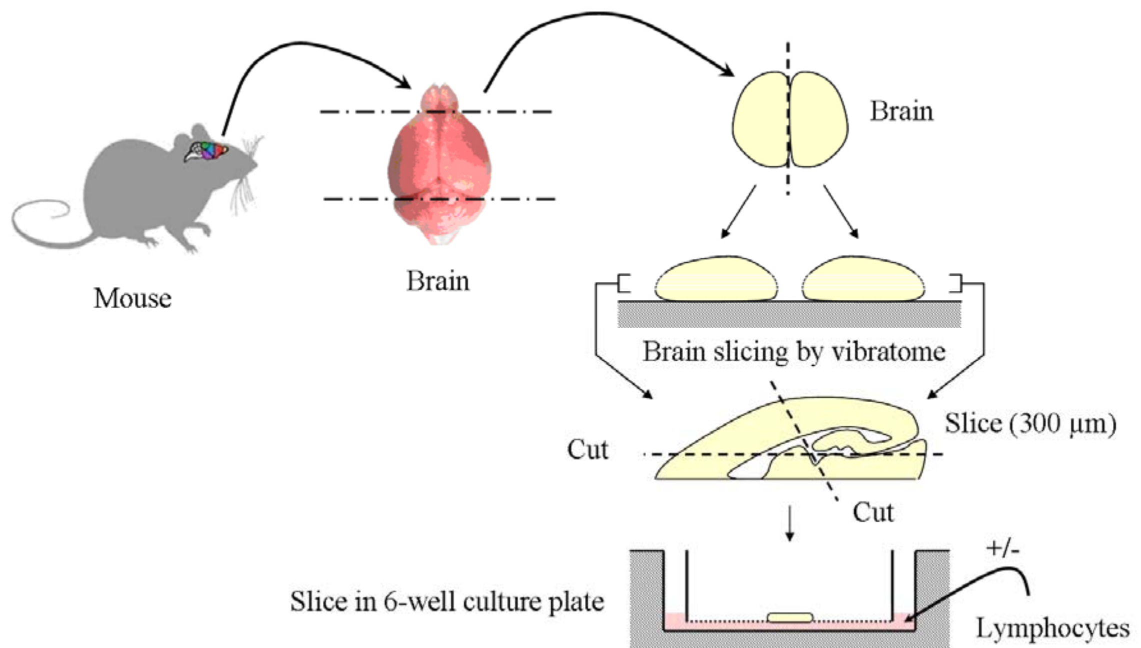


Figure 2.1: Schematic diagram of preparation of Organotypic Slice culture.

The mouse brain is bisected and glued down on a bathing plate. Using a vibratome, parasaggital slices (300µm) were cut and the hippocampus along with the cortex dissected out under binocular microscope using a scalpel blade. Slices were cultured in a 6-well plate on insert membranes with pre-conditioned media (1 ml) underneath for 13-15 days maintaining at 37 °C and 5% CO_2. Whenever lymphocytes were used during experiments, they were added beneath slices separated with insert membrane,

2.2.2 Preparation of lymphocytes

2.2.2.1 Preparation of lymphocyte preparations

Peripheral lymph nodes including axillary, brachial, mesenteric, inguinal, lumbar, caudal and popliteal were harvested from either male or female C57Bl/6 adult mice (6-10 weeks) for mature lymphocytes and transferred to complete RPMI 1640 (cRPMI) media. The cRPMI media contains 10% foetal calf serum and 1% L-Glutamine (200 mM stock concentration).

The lymph nodes were homogenised through a cell strainer (40 µm, BD Biosciences, USA) to extract lymphocytes (Figure 2.2). The whole lymph node preparation (subsequently referred to as 'lymphocyte preparations') was transferred to a sterile tube and centrifuged at 300 X g for 5 minutes. These cell pellets were re-suspended in culture media and washed at least 2 times. The final cell suspensions were prepared in cultured media, counted and assessed for viability using trypan blue. Viable cells were counted on a haemocytometer using phase contrast microscope. A final dilution of a million cells per ml was prepared and this number of cells was used in all experiments where lymphocytes were used.

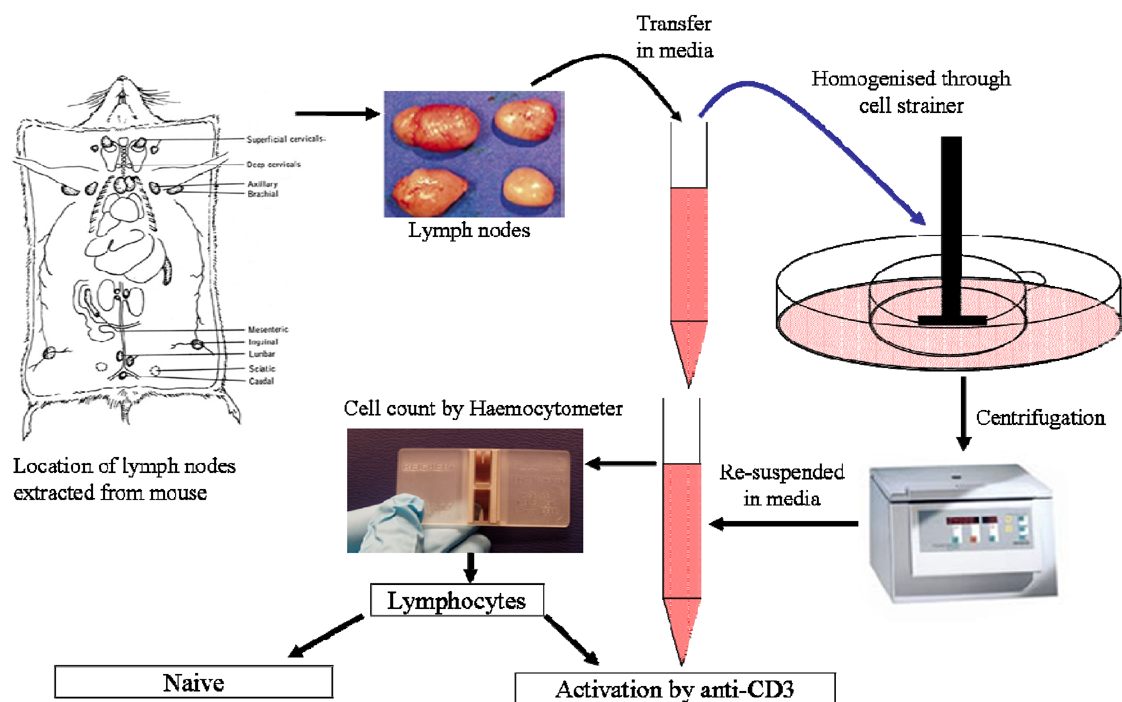


Figure 2.2: Illustration of the process for extraction of lymphocytes from lymph nodes.

Lymph nodes are removed from adult C57Bl6 mice and homogenised through cell strainer. A single cell suspension of lymphocyte preparations were obtained and viable cell count performed using trypan blue to exclude dead or dying cells

2.2.2.2 T cell Purification

Populations of purified T cells were required for certain experiments. Single cell preparations were obtained from lymph nodes as described earlier and cell number determined using trypan blue exclusion for dead or dying cells. T cells were purified from lymphocyte preparations using magnetic-assisted cell sorting (MACS). In MACS, cells of interest are labelled with their specific biotin-conjugated monoclonal antibodies in MACS buffer and further magnetically labelled using anti-biotin-coated, magnetic microbeads which bind to the biotin-conjugated monoclonal antibodies. When these cells run under gravity through a column placed in a strong magnetic field, magnetically labelled cells remain on the column and only unlabelled cells come out from the column. In our study, cells of our interest were not labelled but all other cells were magnetically labelled. This method is referred as a negative selection of cells. MACS buffer was prepared by mixing 0.5% FCS and 2mM ethylene-di-amine tetra-acetic acid (EDTA) in phosphate buffer solution (PBS). T cells were purified by depleting non-T cells using a Pan T Cell Isolation Kit (Miltenyi Biotec, UK) following the manufacturer's instructions and outlined below.

Total lymphocyte cell number was determined and non-T cells were magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies (antibodies against CD11b, D45R, DX5 and Ter-119) as a primary labelling agent while anti-biotin microbeads were used as a secondary labelling agent. All the labelling procedures were performed on ice to avoid any non-specific T cell labelling. The cells were centrifuged (300 X g, 10 mins) and re-suspended in 40 µl of MACS buffer per 10^7 total cells followed by 10 µl of biotin-antibody cocktail per 10^7 total cells. They were mixed well and incubated at 4 °C for 10 minutes. After incubation, 30 µl of MACS buffer and 20 µl of anti-biotin microbeads per 10^7 total cells were added to the mixture, mixed well and incubated at 4 °C for 15 minutes. These cells were washed with MACS buffer by adding 10 to 20 times of labelling volume and centrifuged at 300 X g for 10 minutes. The supernatant was discarded and cells were re-suspended in 500 µl of MACS buffer per

10^8 total cells. These labelled cells were magnetically separated using an LS column placed in the magnetic field of a MACS separator (Milteny Biotec). All labelled cells remain in the column and unlabelled cells (purified T cells) were collected in a sterile tube. The column was washed four times using 3 ml MACS buffer in the magnetic field and collected in the same tube to increase cell yield. The column was then removed from the magnetic field and the column washed using 3 ml MACS buffer to collect labelled cells (non-T cell fraction). The purity of T cells was determined using flow cytometry analysis (FACSCalibur, Becton Dickinson, UK) using anti-CD3e as a primary antibody (see section 2.2.3).

2.2.2.3 Activation of T cells

In experiments where T cell activation was required, non-purified lymphocyte preparations were exposed to anti-mouse CD3e whereas purified T cell preparations were exposed to both anti-mouse CD3e and anti-mouse CD28 (see section 1.3.2). For non-purified lymphocyte preparations, a concentration of 1 μg/ml anti-CD3e was prepared in PBS as described earlier (Wiendl *et al.*, 2003; Grierson *et al.*, 2005) and used to coat a 6-well culture plate by adding 2 mL of this solution per well and incubating for 3 hours at 37 °C, 5% CO_2. This allows the anti-CD3 antibody to adhere to the surface of the culture plate which helps in immobilization of the cell for the interaction. After incubation, wells were washed with PBS and 2 ml of cell suspensions (3 million cells per ml) in cRPMI were added to each well. This culture plate was incubated for 24 hours at 37 °C, 5% CO_2.

For activation of purified T cells, 2 μg/ml of anti-CD3e and 3 μg/ml of anti-CD28 were used as these concentrations were found to be optimum. Anti-CD28, a co-stimulatory factor, was used as a soluble antibody by adding directly into the cell suspension in cRPMI. The cell suspension (3 million cells per ml) was transferred into 6-well culture plates pre-coated with anti-CD3e and incubated for 24 hours at 37 °C, 5% CO_2. After 24

hours, the cell preparations were collected and centrifuged at 300 X g for 5 minutes. Cells were twice washed with culture media, re-suspended in culture media, counted and diluted to 1 million cells per ml for further experiments. Flow cytometry was performed to determine the purity of T cells using CD4 and CD8 and percentage of activated T cells using CD69 and CD62L.

2.2.3 Flow-cytometric Analysis

Flow cytometric analysis was performed using a flow cytometer. The basic principle of flow cytometer is to quantify multiple fluorescence signals from an individual particle and send this signal to the computer for the data processing. A flow cytometer comprises of flow chamber, photomultiplier tubes (PMTs), laser source, filters and computer. The sample is run through the flow chamber where a laser beam interrogates the cells as they pass under laminar flow. This laser light is scattered according to the morphology of each different cell in the cell sample. The light which reflects at right angle is termed as side scattered light whereas light having small angle of reflection is known as forward scattered light. The various types of reflected light will be detected by PMTs and send the data to the computer for the processing. The forward scattering of light represent the size of the cell and side scatter of the light show whether the cells are granulated. Each immune cell has a unique forward and side scatter profile and can be distinguished in analysis accordingly. Additionally, light emitted from specific fluorochromes passes through various filters to further detectors (PMTs), and this information is also sent to the computer. Data is presented graphically as a plot diagram or a histogram following analysis in FlowJo 7.2.4 (Ashland, USA) (Jaroszeski and Radcliff, 1999)

Using a flow cytometer the following was carried out;

- Determine percentage of $CD4^{+/}CD8^{+}$ lymphocytes present in the lymphocyte preparations.
- Confirm activation of T cells following anti-CD3/anti-CD28 stimulation.

- Investigate whether lymphocyte preparations can be activated in serum-free media.
- Determine the purity of T cells after isolation.
- Investigate the B cell population in T cell-depleted lymphocyte preparations.

For flow cytometric analysis, a single cell suspension was prepared and 200 µl was transferred to the FACS tube with at least 100,000 total cells. 1 ml of FACS buffer (0.05% Azide and 2% FCS in PBS) was added and the cells centrifuged at 450 X g, 5 minutes at 4 °C. The supernatant solution was discarded and cells were re-suspended in 100 µl of FcR blocking buffer to avoid any non-specific binding by Fc receptors. FcR blocking buffer was prepared in the immunology laboratory of University of Glasgow by mixing supernatant from anti-CD16/32 hybridoma (2.4G2), 10% mouse serum and 0.1% azide. These cells were then incubated in the dark for 10 minutes at 4 °C. All the relevant monoclonal antibodies and their isotype controls were prepared in Fc block at 1:100 dilutions and added to the tube making the final monoclonal antibodies dilution of 1:200. The cells were incubated in the dark for 20 minutes at 4 °C. These cells were washed twice with 2 ml of FACS buffer and incubated with fluorochrome-cojugated streptavidin (dilution of 1:200) in the dark for 20 minutes at 4 °C wherever appropriate. These cells were washed twice with 2 ml of FACS buffer and re-suspended in 200 µl of FACS flow. In prior to analyse, cells were passed through Nitex mesh (100 µm; Cadisch and Sons, London, UK) to remove any cell clumps. These fluorochrome-labelled cells were then analysed using flow cytometry and data analysed using CellQuest software (BD).

Before acquiring data, forward scatter (FSC) and side scatter (SSC) were adjusted to gate cells of interest. Unstained cells were used as control for auto-fluorescence background. Unstained cells were compared with isotype control and labelled cells to confirm the correct scale. Isotype control antibodies do not have specificity for the target

cells but retain non-specific characteristics of antibodies used within a particular experiment. Therefore, these antibodies are used to confirm the specificity of primary antibodies binding and exclude any non-specific binding resulted from Fc receptor binding. Compensation settings were adjusted using fluorescence channels (FL1, FL2, FL3 and FL4). FL1 positive cells were aligned vertically with negative cells and FL2 positive cells were horizontally aligned in terms of FL1 versus FL2 dot plot. Similar processes were carried out for FL2 versus FL3 and FL3 versus FL4 channels.

2.2.4 Lymphocyte viability in serum-free media

A single-cell suspension was prepared after harvesting from lymph nodes. Cells were cultured in either serum-containing media (cRPMI) or serum-free culture media (Neuorbasal-A/B-27) at 37 °C, 5% CO_2. Cell viability was assessed by counting cells at different time points (1 hr, 3 hrs and 18 hrs) using trypan blue solution.

2.2.5 Cell death assay

2.2.5.1 Concentration-response curve for KA-induced neurotoxicity.

Cell death was induced using Kainic acid (KA) of different concentrations (3 µM to 3 mM) prepared in culture media (Liu *et al.*, 2001). OSCs were incubated with KA at 37 °C, 5% CO_2 for 1 hour. After 1 hour, slices were transferred into the fresh pre-equilibrated culture media containing the fluorescent dye, propidium iodide (2 µM) and incubated for 18 hours at 37 °C, 5% CO_2. PI is a non-toxic dye which enters the cell via damaged cell membrane in dead cells and binds to nucleic acid giving out red bright fluorescence (Macklis and Madison, 1990; Noraberg *et al.,* 1999). This dye is widely used to identify dead cells in cell cultures and in tissue cultures. However, PI cannot differentiate between necrotic and apoptotic cell death. Cells undergoing apoptosis at later stage lysed and release nuclear contents which can bind with PI to give red bright fluorescence (Riccardi and Nicoletti, 2006). After 18 hours, these slices were transferred

to the microscope-mounted incubator chamber (37 °C; Solent Scientific) and PI fluorescence images were captured using a Nikon Eclipse TE 300 inverted epi-fluorescent microscope (4x objective; Ex: 575/25 nm; Em: 626/28 nm; Figure 2.3) equipped with a Hamamatsu CCD camera controlled by MetaMorph software, ver. 7.1.0.0.

The images were adjusted for the equivalent signal distribution using Adobe Photoshop 7.0 and ImageJ (version 1.4.3.67) was used to quantify the fluorescent signal of PI images. The fluorescent signal was expressed as the ratio of integrated density to area of interest as described earlier (Greenwood and Bushell, 2010) and the mean fluorescent intensity indicated average cell death induced by neurotoxic insults including KA and oxygen-glucose deprivation.

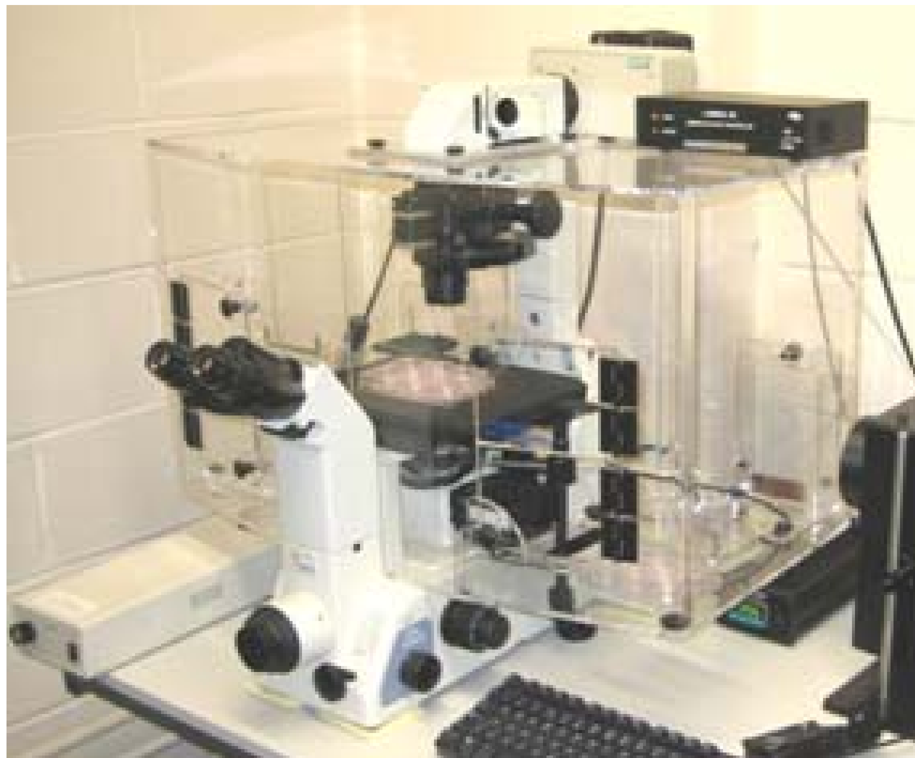


Figure 2.3 Epi-inverted fluorescence microscope mounted with incubator chamber.

2.2.5.2 *Co-culture of OSCs with lymphocyte preparations*

The effect of lymphocyte preparations on OSCs on their own was assessed by co-culturing OSCs with lymphocyte preparations, containing either non-activated or activated T cells. OSCs were incubated with pre-equilibrated culture media containing lymphocyte preparations and PI (2 μM) for 18 hours. OSC incubated in culture media only was taken as a negative control while OSC pre-treated with KA (300 μM) for 1 hour was considered as a positive control for this experiment. In all experiments lymphocytes were placed underneath OSC separated with Millicell membrane insert and were not in direct contact with OSCs. The PI fluorescence image was taken and quantified as explained in section 2.2.5.1.

2.2.5.3 *Co-culture of slices with lymphocyte preparations in the presence of KA*

The effect of lymphocytes on KA-induced neurotoxicity was analysed by co-culturing OSCs and lymphocyte preparations with KA (300 μM) for 1 hour before transfer to fresh culture media containing PI (2 μM) for 18 hours of recovery. Alternatively, OSCs were pre-treated with KA (300 μM) for 1 hour before transfer to pre-equilibrated fresh culture media or pre-equilibrated fresh culture media containing lymphocyte preparations (1 million cells per ml) for the recovery. In all experiments, KA (300 μM) was prepared in culture media wherever it was used.

Various conditions of culture media (listed below) were used after KA treatment.

- Fresh culture media (positive control)
- Culture media containing lymphocyte preparations either with non-activated or activated T cells
- Culture media containing purified T cells or T cell-depleted lymphocyte preparations (Non-T cells)

PI (2 μM) was added in all conditions and fluorescence images were taken and

quantified as described in section 2.2.5.1.

2.2.6 Cell death assay using conditioned media

2.2.6.1 Preparation of conditioned media

To investigate the lymphocyte-dependent response and the possible effects of soluble factors on KA-induced cell death in OSC, five types of conditioned media were used as treatments: conditioned media with exposure to OSC, conditioned media without exposure to OSC, conditioned media from OSC exposed to KA only, conditioned media from rat primary neuronal culture and heated conditioned media.

2.2.6.1.1 With exposure to OSC

Lymphocytes were prepared as described in section 2.2.2.1 and incubated in cRPMI for 24 hours in 37 °C, 5% CO_2. After 24 hours, cells were collected in a sterile tube and centrifuged at 300 X g for 5 minutes. These cell pellets were washed twice with culture media and re-suspended in culture media. This cell suspension was incubated in 37 °C, 5% CO_2 for 2 hours in order to equilibrate. OSCs were treated with KA (300 μM) for 1 hour and transferred to culture media containing lymphocyte preparations (1 million cells per ml). After incubating for 18 hours, media was removed and transferred to a sterile tube. Cells were centrifuged at 700 X g for 10 minutes and the supernatant was collected. This supernatant was incubated at 37 °C, 5% CO_2 for 1 hour in order to equilibrate and used as ‘conditioned media-I’.

2.2.6.1.2 Without exposure to OSC

Lymphocyte preparation was prepared as described in section 2.2.2.1 and incubated in cRPMI for 24 hours in 37 °C, 5% CO_2. After 24 hours, cells were collected in a sterile tube and centrifuged at 300 X g for 5 minutes. These cell pellets were washed twice with culture media and re-suspended in culture media. This cell preparation (1 million cells

per ml) was further incubated for 18 hours in 37 °C, 5% CO_2. After 18 hours, cells were collected in a sterile tube and centrifuged at 700 X g for 10 minutes. The supernatant was collected and incubated at 37 °C, 5% CO_2 for 1 hour for equilibrium. This supernatant was used as a 'conditioned media-II'.

2.2.6.1.3 From OSC exposed to KA only

OSCs were treated with KA (300 μM) for 1 hour and transferred to pre-equilibrated fresh culture media. After incubating for 18 hours, media was removed and centrifuged at 700 X g for 10 minutes. The supernatant solution was collected and incubated at 37 °C, 5% CO_2 for 1 hour in order to equilibrate. This supernatant was used as a conditioned media from KA, termed 'conditioned media-III'.

2.2.6.1.4 From rat primary hippocampal neuronal culture

Primary hippocampal neuronal cultures were prepared from 1-2 days old Sprague-Dawley rat as described previously (Gan *et al.*, 2011) in the laboratory by my colleague, Conor Doherty. When cultures were 2-3 days old, media was changed with fresh culture media and incubated for 18 hours. After 18 hours, media was removed and centrifuged at 700 X g for 10 minutes. The supernatant solution was collected and incubated at 37 °C, 5% CO_2 for 1 hour in order to equilibrate which was used as 'conditioned media-IV'.

2.2.6.1.5 Heated Conditioned media

Conditioned media-II was prepared as described in section 2.2.6.1.2. This media was maintained at temperature of 74 °C for 15 minutes to denature proteins. The heat-treated conditioned media was then cooled to 37 °C in an incubator to allow temperature and pH equilibration.

2.2.6.2 Cell death assay

OSCs were treated with KA (300 μM) for 1 hour and transferred to conditioned media with PI (2 μM). They were incubated in 37 °C, 5% CO_2 for 18 hours and PI fluorescence images were taken and quantified as described in section 2.2.51.

Various experimental conditions were used after pre-treatment which are listed below;

- Conditioned media-I
- Conditioned media-II
- Conditioned media-III
- Heated conditioned media
- Conditioned media-IV

2.2.6.3 Oxygen-glucose deprivation (OGD) model of experimental ischaemia.

Normally, artificial cerebro-spinal fluid (aCSF) contains 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 2.5 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose but for oxygen-glucose deprivation condition, glucose was replaced by sucrose in aCSF. The concentration of oxygen was minimised as far as possible by continuous bubbling with nitrogen at least for 2 hours. OSCs were transferred to the plate containing 1 ml of OGD-aCSF underneath of the insert membrane with a further 1ml of OGD-aCSF added on top of the slice and incubated in a nitrogen chamber for 30 minutes. The slices were then transferred to the culture media containing PI (2 μM) and incubated for 18 hours for the recovery. The PI fluorescence images were taken and quantified as described in section 2.2.51.

2.2.7 SDS-PAGE and Western blotting

2.2.7.1 Preparation of samples

When OSCs were 13-15 days old, they were transferred to media containing lymphocytes and incubated for 18 hours while the control slices were incubated in fresh media. Following this incubation, the plate containing OSCs was placed on ice and the media replaced with ice-cold HEPES-buffered saline (140 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 10 mM HEPES; 10 mM D-glucose; 310 mOsm; pH 7.4). Slices were washed three times with ice cold PBS, keeping PBS above and below the insert membrane. OSCs were cut out from the insert with membrane attached and kept in solubilisation buffer [0.3% (v/v) NP-40; 25 mM Tris; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 150 mM NaCl; 10 µg/ml leupeptin; 10 µg/ml aprotinin; 10 µg/ml pepstatin A; 0.4 mM phenylmethylsulfonyl fluoride (PMSF); 1 mM Na_3VO_4; pH 7.4] at least for 1 hour on the ice with occasional vortexing. When the OSCs detached from membranes, the membranes were removed and slices were triturated with a needle and syringe at least four times. The total cellular protein was determined by Bradford reaction and normalised to 0.444 µg/µL with solubilisation buffer. Samples were further diluted by 25% with sample buffer [63 mM Tris; 50 mM dithiothreitol (DTT); 5 mM ethylenediamine-tetra-acetic acid (EDTA); 2 mM $Na_4P_2O_7$; 10% glycerol; 2% sodium dodecyl sulfate (SDS); 0.007% bromophenol blue; pH 6.8] and boiled for 5 minutes to denature proteins.

2.2.7.2 Preparation of Polyacrylamide gels

Glass plates were cleaned with ethanol and assembled in the appropriate frame. SDS-PAGE is made up of two different layers, a resolving layer and a stacking layer. For each resolving layer, approximately 4.5 ml of 10% resolving gel was prepared. A 10% resolving gel, which is enough for two gels, contains 4.1 ml of water, 3 ml of Buffer 1 [1.5 M TRIZMA base; 0.4%(w/v) SDS; pH 8.8], 4 ml of Acrylamide [30% (w/v) acrylamide; 0.8% (w/v) bis-acrylamide], 0.8 ml of 50% (v/v) glycerol, 45 µl of 10%

(w/v) ammonium persulphate (APS) and 4 µl of tetramethylethylenediamine (TEMED). TEMED acts as a polymerising agent so it was added last to the mixture. This resolving gel was poured between the assembled glass plates and allowed to set for at least 1 hour overlaying 0.1% (w/v) SDS on top of the gel. When the resolving gels were set, stacking gels (approximately 2.5 ml per gel) were prepared. For each stacking layer, approximately 2.1 ml of stacking gel was prepared. A stacking gel (enough for 2 gels) contains 4.88 ml of water, 1.88 ml of Buffer 2 [0.5 M TRIZMA; 0.4% (w/v) SDS; pH 6.8], 0.75 ml of Acrylamide [30% (w/v) acrylamide; 0.8% (w/v) bis-acrylamide], 75 µl of 10% (w/v) APS and 10 µl of TEMED. The SDS was discarded and the stacking gels poured on top of the resolving gels. The comb was inserted in the stacking gels and everything allowed to set at least for 30 minutes. When the stacking gel was set, the comb was removed and the lanes washed three times with running buffer (24.76 mM TRIZMA base, 191.8 mM Glycine and 3.46 mM SDS).

2.2.7.3 Polyacrylamide gel electrophoresis

These gel cassettes were assembled into a BIO-RAD Mini-PROTEAN 3 Cell electrophoresis tank and the tank was filled with running buffer. Pre-stained SDS-PAGE molecular weight marker (15µl) was loaded into the first well. This is a reference standard for the molecular weight of samples. 30 µl of samples were loaded into wells of stacking gel using a Hamilton syringe. After loading samples, the 10% SDS-polyacrylamide gel electrophoresis was run at 130 volts at least for 1 hour and 30 minutes or until the bromo-phenol markers in samples reached the bottom of gel. The protein molecules get separated on the basis of size, electric charge and other physical properties by the effect of electric field.

2.2.7.4 Transfer to nitrocellulose

When protein bands were resolved by SDS-PAGE, glass cassettes were disassembled and gels were transferred to a nitrocellulose sheet using BIO-RAD Mini Trans-Blot Kit

immersed in transfer buffer (30.9 mM TRIZMA base, 239.7 mM Glycine and 25% (v/v) Methanol). The transfer cassettes were assembled in the following sequential order; sponge, blotting paper, gel, nitrocellulose, blotting paper and sponge. These transfer cassettes were assembled into BIO-RAD Mini-PROTEAN 3 Cell electrophoresis tanks, filled with transfer buffer was and run for at least for 1 hour and 45 minutes at 240 mA.

2.2.7.5 Western blotting

When samples were blotted to nitrocellulose, the transfer cassettes were disassembled and nitrocellulose was transferred to dish containing blocking solution to prevent from unspecific binding. The blocking solution is made up of 3% (w/v) BSA in wash (NaTT) buffer (150mM NaCl; 50mM Tris; 0.2% Tween 20; pH 7.4). The nitrocellulose was kept in the blocking solution at least for 2 hours at room temperature on orbital shaker (THERMO Electron Corporation) at speed of 50-55 rpm. After 2 hours of blocking, blots were probed with the specific primary antibodies for phosphorylated proteins (ERK, p38 and JNK) [1:5000 dilutions for p-ERK in 0.2% (w/v) BSA/NaTT and 1:7500 dilution in 0.2% (w/v) BSA/NaTT for p-p38 and p-JNK] and incubated overnight at room temperature on an orbital shaker at 50-55 rpm. The next day, unbound antibodies were removed with wash buffer for 2 hours changing buffer every 15 minutes. The blots were then probed with horse-radish-anti-mouse IgG or anti-rabbit IgG secondary antibodies [1:6667 dilutions in 0.2% (w/v) BSA/NaTT] for 1 hour and 30 minutes at room temperature on the orbital shaker. The blots were again washed with NaTT buffer for 2 hours changing buffer every 15 minutes. Finally, blots were placed in a mixture (1:1 dilution) of enhanced chemiluminescence (ECL)-1 (1 M Tris; 250 mM Luminol; 250 mM p-Cumaric acid; pH 8.5) and ECL-2 (1 M Tris; 0.064% hydrogen peroxide; pH 8.5) reagents for 5 minutes. After draining reagents, the blots were kept in between two sheets of clean transparent plastic film in an exposure envelope. X-ray film was exposed to the blot for 5 minutes or less than 5 minutes (depending on the intensity of the band) inside a dark room and developed using X-OMAT developing machine. The phosphorylated proteins were detected by phospho-specific bands.

2.2.7.6 Stripping and reprobing of nitrocellulose membranes

This step is necessary to ensure that the equal amount of protein from each sample was loaded in the gel. To determine total protein, blots from the previous western blotting were stripped using stripping buffer (100 mM 2-mercaptoethanol; 62.5 mM Tris; 2% SDS; pH 6.7). The blots were incubated in stripping buffer for 1 hour at 75^0 C on orbital shaker at a speed of 55 rpm. The blots were washed with wash buffer for 30 minutes, changing buffer every 10 minutes. The blots were then re-blocked for 2 hours in blocking solution changing the solution every 15 minutes. Blots were re-probed with non-phospho-specific primary antibodies [1:7500 dilutions in 0.2% (w/v) BSA/NaTT] and incubated overnight on an orbital shaker. On the following day, the blots were washed as described above and probed with respective secondary antibodies (1:6667 dilutions) for 1 hour and 30 minutes. The blots were washed again with NaTT buffer for 2 hours, changing buffer every 15 minutes before developing the bands as explained above. Both phospho-specific and non-phospho-specific bands were scanned and quantified using ScnImage (NIH).

2.2.8 Pharmacological treatment of OSCs

Various drugs were used in the presence or absence of conditioned media in KA-induced cell death and OGD induced cell death. In KA-induced cell death, OSCs were pre-treated with KA for 1 hour and incubated in either fresh culture media or different condition of conditioned media (listed below) for 18 hours. Similarly, in OGD induced cell death, after exposing OSCs to OGD, OSCs were transferred to either fresh culture media or various conditions of conditioned media (listed below) and incubated for 18 hours. PI (2 µM) was used in both conditions. After 18 hours, the PI fluorescence images were taken and quantified as described in section 2.2.51.

Various conditions of media are listed below;

- Fresh culture media (positive control) with PI

- Fresh culture media containing SB203580 (100 μM), a p-38 MAP kinase inhibitor and U0126 (20 μM), a MEK/ERK inhibitor along with PI
- Conditioned media-II containing LY294002 (10 μM), a phosphotidyl-inositol-3 (PI-3) kinase inhibitor and PI: In the KA model, slices were treated with KA (300 μM,1 hr) in the presence of LY294002 and incubated in conditioned media-II in the presence of LY294002 for 18 hours. Similarly, in OGD model, slices were exposed to OGD for 30 minutes in the presence of LY294002 and incubated in conditioned media-II in the presence of LY294002 for 18 hours.
- Conditioned media-II containing fluoroacetate (FAc, 10 μM), a glial cell metabolic inhibitor and PI: In KA model, slices were pre-treated with FAc for 2 hours and 1 hour further treated in the presence of KA (300 μM) while slices were pre-treated with FAc for 2 hours 30 minutes and further treated with FAc for further 30 minutes in OGD condition for OGD model and incubated in conditioned media-II containing FAc for 18 hours.

2.2.9 Immunohistochemistry

Immunohistochemistry protocol was followed using a previously described method (Gogolla *et al.*, 2006) with few modifications. For immunofluorescnce imaging of T cell infiltration into slices, lymphocyte preparations were prepared from peripheral lymph nodes of mice expressing green fluorescent protein (GFP) under the human CD2 promoter (resulting in GFP expression by peripheral T cells). OSCs were incubated with lymphocyte preparations containing GFP-expressing T cells for 18 hours after treatment with or without KA for 1 hour. After 18 hours of recovery, the media was removed by suction and 1 ml cold 4% paraformaldehyde (PFA) was added above and below the insert membrane for 5 minutes. OSCs were washed twice with cold PBS and 100% methanol was then added above and below the membrane for 10 minutes, after which OSCs were washed with PBS. OSCs were incubated overnight with permeabilization solution (0.5% Triton X-100 in PBS) before blocking with 20% Bovine serum albumin

in PBS for 4 hours at room temperature. The OSCs with insert membrane was cut-off and placed facing topside up to minimize the required volume of antibody. To identify neurons, a mouse anti-microtubule-associated protein 2 (anti-MAP2; 1:5000) was used as a primary antibody and anti-mouse Alexa Fluor 555 anti-mouse IgG (1:200) was used for a secondary antibody. For GFP-expressing T cells, a rabbit anti-GFP (1:400) was used as primary antibody and Alexa Fluor 488 anti-rabbit IgG (1:200) used as a secondary antibody. Further, to investigate microglia, rat anti-mouse CD11b (1:100) was used as a primary antibody and Alexa Fluor 633 anti-rat IgG (1:200) used as a secondary antibody. OSCs were incubated overnight with primary antibodies at 4 °C and washed 3 times with 5% bovine serum albumin (BSA)/PBS. The secondary antibodies were added to OSCs and incubated for 3 hours at room temperature. OSCs were washed with 5% BS/PBS for 3 times and mounted on the glass microscope slide. A drop of Vectashield (a mounting medium for fluorescence) with 4',6-diamidino-2-phenylindole (DAPI) was added to each OSC and a thin glass cover slip was used to cover OSCs. The cover slips were sealed using nail polish. Those cover slips were stored at 4 °C until images were captured using a Leica TCS SP-5 laser scanning confocal system with a 40x oil immersion lens. A Z-stack of images (approximately 42 of 4-5 µm thickness) was taken and was optimised using Adobe photoshop (version 7.0). For the positive control, a million cells were added on the top of the slice as opposed to the usual arrangement of T cells underneath the transwell membrane and for the negative control, only secondary antibodies were added to the slice without adding primary antibodies to avoid any background fluorescence from non-specific bindings and auto-fluorescence.

2.2.10 Statistical Analysis

All the data are presented as mean ± S.E.M. from the number of slices indicated in all experiments performed on at least three different culture preparations. At least six slices were obtained from each mouse and each experiment was carried out in at least three separate animal preparations. Positive controls (slices treated with KA 300 µM for 1 hour or exposed to OGD for 30 mins) were performed in every experiments and slices

treated in various conditions were compared with the positive control. The differences between data were analysed using a one-way ANOVA with Tukey's post-hoc analysis unless otherwise stated. In all analyses, $p < 0.05$ were considered as significant (Prism version 4.03).

3 Development of Organotypic slice culture for experimental model of neurodegenerative diseases

As my research is to investigate the role of lymphocytes in neurodegenerative diseases, it is important to develop a viable experimental setup to test the research hypothesis. I therefore, performed a series of control experiments to setup the viable experimental model which I have described in this chapter.

3.1 Introduction

Organotypic slice culture (OSC) has been a widely used tool to investigate a variety of parameters such neurogenesis, regeneration and response to trauma *in vitro* (Krassioukov *et al.*, 2002; Rambani *et al.*, 2009). This method is also well established as a model to study cellular and molecular mechanisms of neuronal death, synaptic plasticity and neuroprotection (Gähwiler *et al.*, 1997; Holopainen, 2005). OSC also retains the cytoarchitecture of intact brain tissues which are more likely to reflect *in vivo* situations and has been extensively used as an *in vitro* model for neurodegenerative diseases including stroke to investigate pathology of these diseases and related signalling mechanism(s) (Liu *et al.*, 2001; Krassioukov *et al.*, 2002; Fujimoto *et al.*, 2004; Zamin *et al.*, 2006; Greenwood and Bushell, 2010). It has previously been demonstrated that OSC can be cultured in serum free culture media (Brewer and Cotman, 1989; Brewer *et al.*, 1993; Huuskonen *et al.*, 2005; Greenwood and Bushell, 2010). OSC cultured in serum-free media maintain morphological integrity and respond normally to pro- and anti-inflammatory stimuli (Huuskonen *et al.*, 2005). Further, culturing in a serum-free media is useful when it is required to control the levels of growth factors, hormones and amino acids (Brewer *et al.*, 1993) since, serum itself can induce glial cells to release inflammatory mediators and inflammation-related enzymes (Si *et al.*, 2000; Cooper *et al.*, 2002). In the present study, OSCs were cultured in serum-free culture media and the role of lymphocytes was investigated in two different models of neurodegenerative disease: kainate-induced cell death and oxygen-glucose deprivation (OGD) induced cell death, an *in vitro* model for stroke.

Kainate ((2S, 3S, 4S)-3-(carboxymethyl)-4-(prop-1-en-2-yl) pyrrolidine-2-carboxylic acid) is obtained from algae called Digenea simplex. It has been suggested that kainate (KA) induces cell death via kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Nielsen and Liljefors, 2001). Kainate has a high affinity for kainate receptors whereas it has a lower affinity to AMPA receptors by a factor of 600 (Huettner, 1990; Conti *et al.*, 1999; Nielsen and Liljefors, 2001). Kainate receptors are widely distributed in the CNS and are considered as one of the determinant causing neurodegenerative diseases (Monaghan and Cotman, 1982; Coussen, 2009). The mechanism of excitatory cell death induced by kainate has been suggested to be via stimulating AMPA/KA receptors that leads to an increase in intracellular calcium levels and this calcium-dependent pathway leads to neuronal apoptosis (Wang *et al.*, 2005). The binding of kainate to AMPA/KA receptors cause depolarisation leading to activation of voltage-gated calcium channels, influx of extracellular calcium and release of glutamate (Ferkany *et al.*, 1982; Zheng *et al.*, 2011). This depolarisation further activates voltage dependent N-methyl D-aspartate (NMDA) receptors by removal of magnesium ions (Mg^{2+}) block in the presence of glutamate leading more calcium influx and causing neuronal toxicity (Mayer *et al.*, 1984; Nowak *et al.*, 1984; Sattler and Tymianski, 2001; Blanke and VanDongen, 2009). Kainate toxicity has been suggested to be dependent on influx of extracellular calcium ions (Garthwaite and Garthwaite, 1986; Rothman *et al.*, 1987; Cheng and Sun, 1994) and intracellular overload of calcium ions (Wang *et al.*, 2005) leading to neuronal death. Furthermore, it has also been suggested that kainate causes the release of reactive oxygen species causing dysfunction of mitochondria, ultimately leading to apoptosis in neurons (Sun *et al.*, 1992; Cheng and Sun 1994). CNS oxidative stress has been proposed to be a major cause of neuronal death in the kainate model of experimental epilepsy (Gluck *et al.*, 2000). Excitotoxic-mediated neuronal death is one of the key features implicated in a number of CNS pathologies like stroke and PD (Choi, 1996; Choi *et al.*, 2005). Moreover, it has been suggested that KA-induced neuronal death can be a model for neurodegeneration (Wang *et al.*, 2005). Though KA-induced cell death can be considered as a model for

neurodegeneration, its effect in biological systems can be varied. Therefore, the present study has investigated the effect of various concentrations of KA in murine OSCs to validate a model of neurodegeneration.

In addition, it is also vital to know the physiological response of OSCs to lymphocytes under the present experimental conditions. These cells are involved in the adaptive immune system and initiate immune reactions in response to pathogens or inflammatory conditions. As described in Chapter 1, various studies have demonstrated that lymphocytes can migrate into the CNS irrespective of their antigen specificity (Wekerle *et al.*, 1991) in healthy conditions as well as in disease states (Hickey *et al.*, 1991; Hickey, 2001; Ransohoff *et al.*, 2003; Engelhardt, 2010). These cells regularly patrol the brain and scan for their cognate antigens (Hickey, 1991; Engelhardt and Ransohoff, 2005). Those lymphocytes which fail to recognise their cognate antigens regularly exit from the CNS (Hickey *et al.*, 1991). Further, it has been illustrated that the migration of lymphocytes into the CNS depends on their activation state (Hickey *et al.*, 1991; Hirschberg *et al.*, 1998; Hickey, 1999), since various adhesion molecules and chemokines receptors are expressed in activated lymphocytes which enable them to cross the BBB (Kivisäkk *et al.*, 2002; Engelhardt and Wolburg, 2004; Engelhardt, 2006; Reboldi *et al.*, 2009). Therefore, from this evidence it is clear that lymphocytes regularly scan for any antigens under normal conditions. However, there is a lack of evidence for the role of lymphocytes in *in vitro* conditions, including slice cultures. Although, it is important to develop a model for neurodegeneration to investigate the role of lymphocytes in such conditions, it is also equally vital to find out the response of OSCs to lymphocytes in normal OSC culture conditions before investigating the role of lymphocytes in neurodegenerative condition. Hence, the current study has considered KA-induced cell death as a model for neurodegeneration and the present study has investigated the role of lymphocytes in normal OSC culture conditions.

3.2 Results

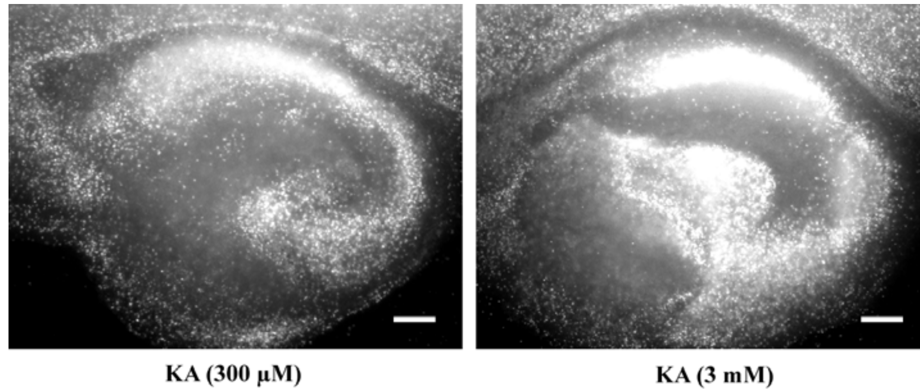
3.2.1 Kainate-induced neurotoxicity in OSCs is concentration-dependent

OSCs were treated with five different concentrations of kainate (KA, 3 μM - 3 mM) to assess the neuronal death pattern. Slices were treated with KA for 1 hour, transferred to fresh culture media containing PI (2 μM) and then incubated for 18 hours.

KA induces neurotoxicity in a concentration dependent manner with a concentration of 3 mM KA inducing maximum neuronal death. All data are expressed in terms of average mean fluorescent intensity, more the fluorescent intensity, more number of cell death. The concentration-dependent manner of neuronal death can be also seen in representative fluorescence images (Figure 3.1 A). The avergae mean fluorescent intensity for 300 μM KA was 45.7 ± 5.3 (n = 35 from 22 animals) and 68.5 ± 5.0 was found for 3 mM KA (n = 15 slices from 5 animals). From the concentration-response curve, the effective concentration for 50 % cell death (EC_{50}) for KA was found to be 190.0 ± 9.0 μM (Figure 3.1 B). The concentration of 300 μM KA was used as a positive control for the rest of my study in this thesis as 300 μM KA didn't induce maximum cell death. The cell death induced by KA (300 μM) was set to 100% and all other values were compared with this concentration.

To clarify that the KA-induced neurotoxicity was mediated via ionotropic glutamatergic receptors as has been reported by previous studies (Wang *et al.*, 2005; Greenwood and Bushell, 2010; Contractor *et al.*, 2011), experiments were performed in the presence of NBQX (20 μM), antagonist for AMPA/KA and DL-AP5 (100 μM), NMDA antagonist. I show that the neurotoxicity induced by KA (300 μM) was completely blocked by NBQX and DL-AP5 (10.2 ± 4.0 % of the 300 μM KA control, n = 6 slices from 4 animals; $p < 0.001$; Figure 3.2).

(A)



(B)

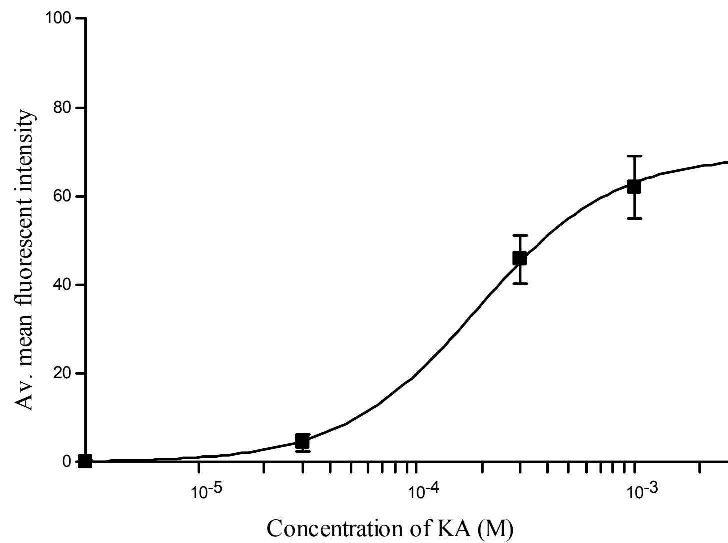


Figure 3.1 KA induces neuronal death in a concentration dependent manner.

(A) Representative PI images showing cell death induced in slices treated with KA (300 μM) or KA (3 mM) for 1 hour and incubated in fresh culture media for 18 hours. (B) Concentration response curve showing cell death induced by KA. All experiments were carried out in at least 4 separate animals. Data presented as mean ± SEM. Scale bar: 200 μm.

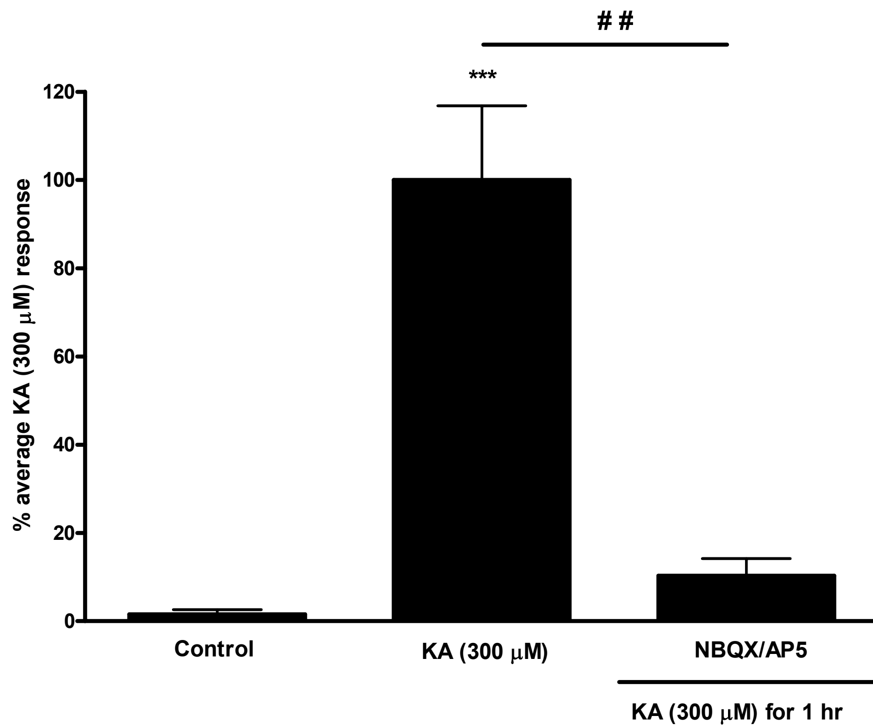


Figure 3.2 KA-induced cell death is mediated via ionotropic glutamatergic receptors.

Bar chart showing the effect of NBQX and AP5 against kainate-induced cell death. Slices treated with KA (300 μM) for 1 hour in the presence of NBQX and AP5 in combination and incubated in fresh culture media for 18 hours reduced KA-induced cell death. All experiments were carried out in at least 4 separate animals. Data presented as mean ± SEM. ##$p < 0.01$ versus NBQX/AP-5 treated slice; ***$p < 0.001$ versus fresh culture media (control).

3.2.2 Lymphocytes can be cultured in serum free culture media as effectively as serum containing cRPMI

To investigate the role of lymphocytes in an *in vitro* model of neurodegenerative diseases, it is crucial to know the response of OSCs to lymphocytes under normal OSC culture conditions. However, initially, it was important to investigate whether lymphocytes can survive in serum-free OSC culture media as lymphocytes are normally cultured in cRPMI, a serum containing media. I therefore assessed the viability of lymphocytes maintained in serum-free OSC culture media with respect to cRPMI. Both types of media (cRPMI and serum free OSC culture media) are equally suitable for lymphocytes cultures by performing cell counts at various time points (Figure 3.3). Following 18 hours in culture, 61.5 ± 8.5 % (n = 4 samples from 4 animals) of lymphocytes survived in serum free OSC culture media while 62.6 ± 9.8 % (n = 4 samples from 4 animals) of lymphocytes were viable in cRPMI which suggests that survival of lymphocytes is not affected by the culture media in which these cells were maintained.

As I need to add KA in lymphocyte preparations for my experiments, I therefore assessed whether adding KA in lymphocyte preparations affect the survival of lymphocytes in serum free OSC culture media. Following the method described earlier (see section 2.2.4), lymphocytes were incubated either in the presence or absence of KA and cell count was done using haemocytometer in two time points (1 hr and 3 hr, Figure 3.4). Following 3 hours in culture, 83.3 ± 3.8 % (n = 7 samples from 7 animals) of lymphocytes survived in the media containing KA while 81.8 ± 4.5 % (n = 4 samples from 4 animals) of lymphocytes were viable in the media without KA which suggests that the presence of KA does not affect the survival of lymphocytes.

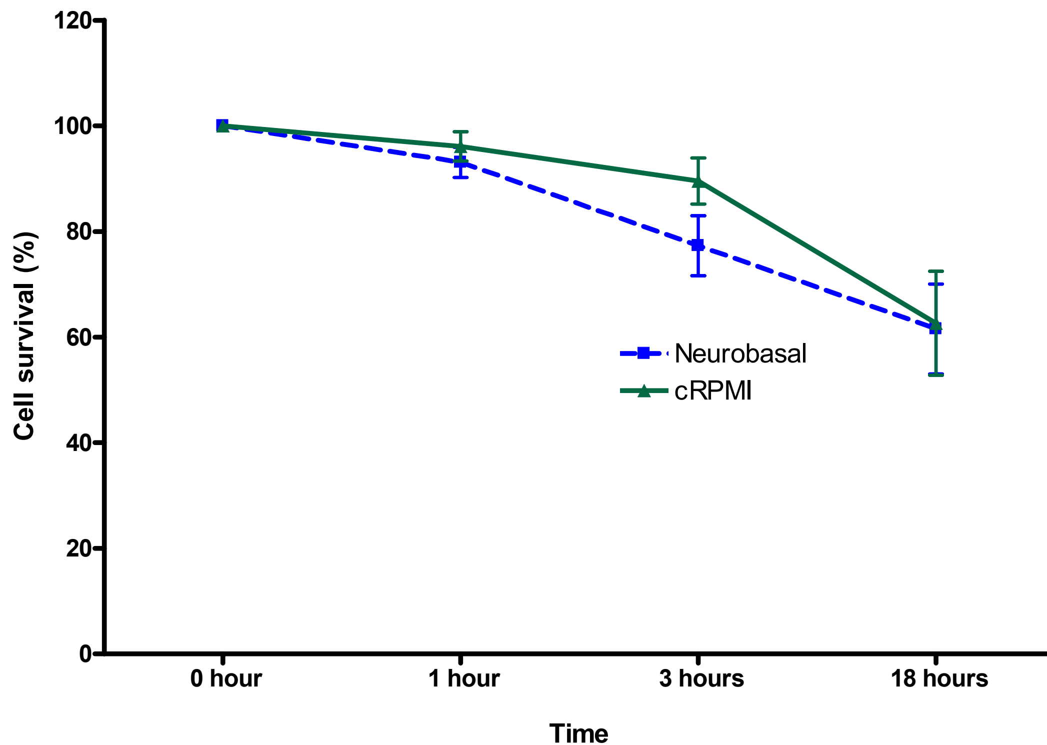


Figure 3.3 Lymphocytes can be cultured in serum free OSC culture media as effectively as cRPMI.

A line graph showing the survival of lymphocytes in two different types of culture media. Lymphocytes were cultured in serum-free OSC culture media (Neurobasal/B-27; blue dotted line) or in serum-containing RPMI (cRPMI; green line) and cell viability measured at various time points using trypan blue counting. All experiments were carried out in at least 4 separate animals. Data were analysed using two-way ANOVA and presented as mean ± SEM.

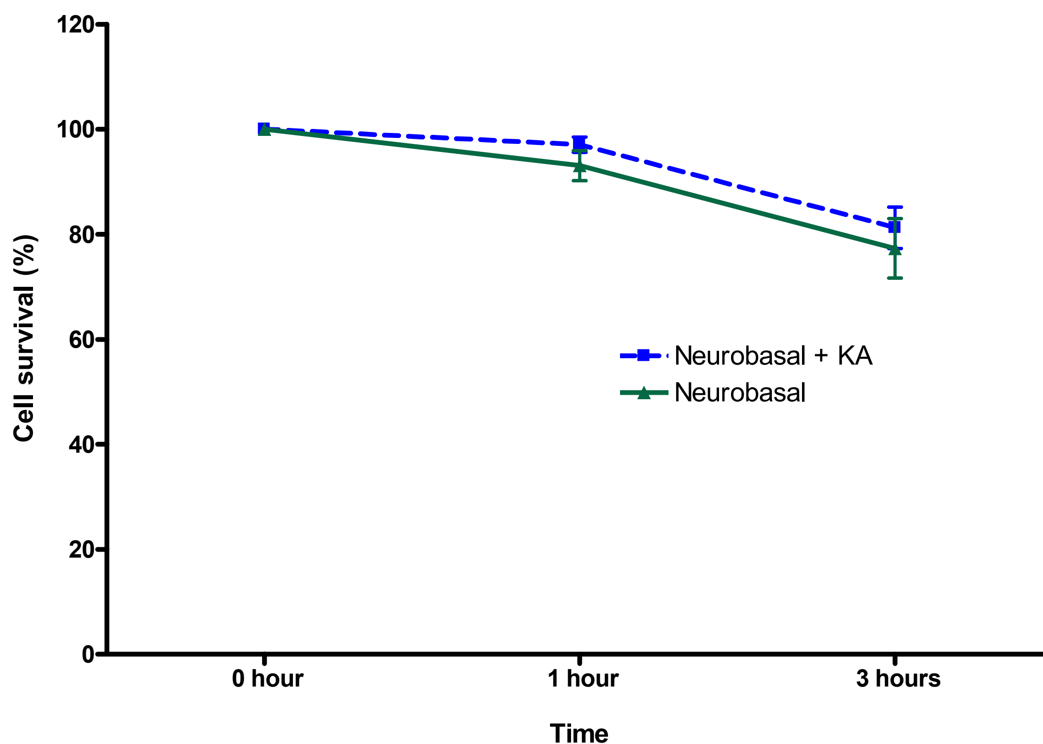


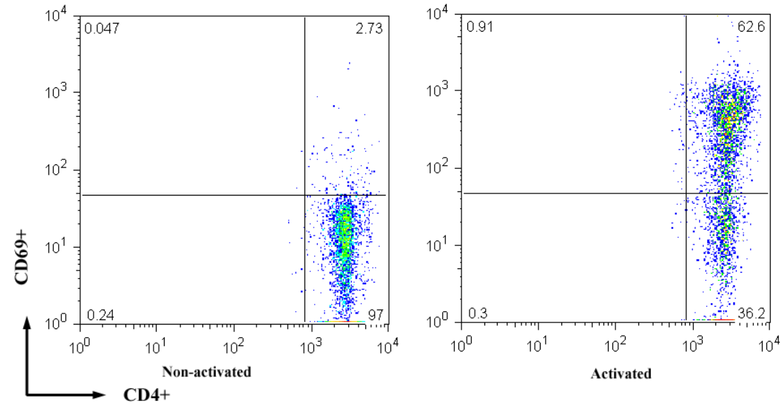
Figure 3.4 KA does not affect the survival of lymphocytes.

A line graph showing the survival of lymphocytes in different time points. Lymphocytes were cultured either in the presence or absence of KA in serum free OSC culture media (Neurobasal-A/B27). Blue dotted line represents Neurobasal-A/B27 in the presence of KA and green solid line represents Neurobasal-A/B27 alone. All experiments were carried out in at least 4 separate animals. Data were analysed using two-way ANOVA and presented as mean ± SEM.

3.2.3 Investigating the activation of T cells in cRPMI and culture media

Having established that T cell viability was not compromised following maintenance in serum-free OSC culture media, I next investigated whether these T cells can also be effectively activated. Lymphocyte preparations were incubated in either cRPMI or culture media along with anti-CD3 for 24 hours to induce T cell activation. The activation state of T cells was then assessed by flow cytometry analysis using CD69 as an activation marker. CD69 is expressed in activated T cells and is routinely used as an activation marker as it is rapidly up-regulated on the cell surface of activated T cells (Cambiaggi *et al.,* 1992; Vassiliadou and Bulmer, 1998). T cell activation was determined by determining the proportion of cells that were $CD4^{+}CD69^{+}$ double positive or $CD8^{+}CD69^{+}$ double positive (Figure 3.5). In cRPMI, 67.6 ± 4.8 % (n = 9 samples from 9 animals) of CD4+ lymphocytes and 48.5 ± 9.1 % (n = 9 samples from 9 animals) $CD8^{+}$ lymphocytes were found to co-express CD69. Similarly, in serum-free OSC culture media, 70.7 ± 7.4 % (n = 5 samples from 5 animals) of $CD4^{+}$ lymphocytes and 52.5 ± 12.7 % (n = 5 samples from 5 animals) of $CD8^{+}$ lymphocytes were activated (Figure 3.6) indicating that T cells can be activated as effectively in serum-free conditions as in cRPMI, a serum-containing media.

(A)



(B)

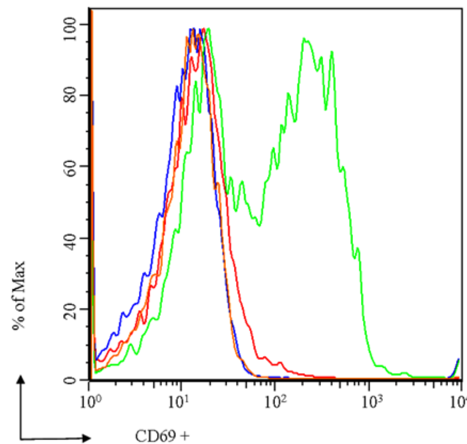
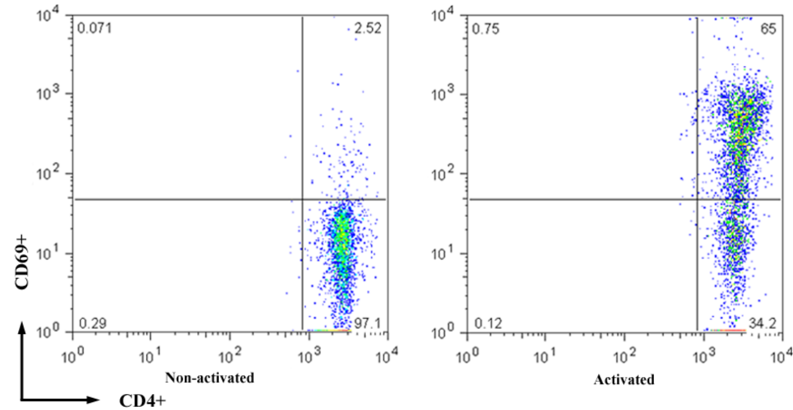


Figure 3.5 T cells are activated in serum containing media.

Cells were incubated with anti-CD3 for 24 hours in serum containing media (cRPMI) and activation of T cells was assessed using anti-CD69 post-incubation. Cells which were incubated for 24 hours without anti-CD3 in similar media were taken as a control. Both types of cells were treated in a similar manner with anti-CD69. (A) Representative dot-plot showing activation of T cells in comparison to their non-activated phenotypes in serum containing media (cRPMI). (B) Representative histogram showing up-regulation of CD69 marker in activated T cells. Green line represents activated T cells and red line represents non-activated T cells while blue line represents isotype control and orange line represents unstained cells. The right hand shift of green graph represents the up-regulation of CD69 marker in comparison to red graph.

(A)



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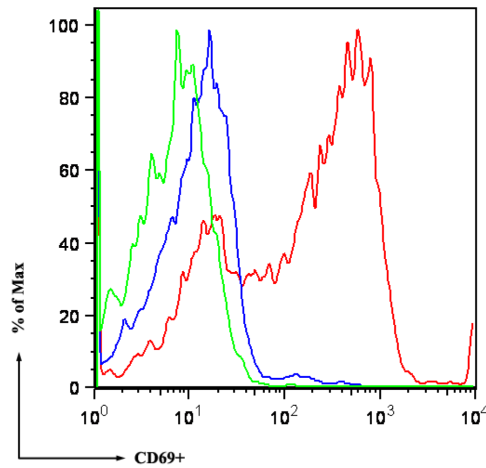


Figure 3.6 T cells can be activated in serum free OSC culture media.

Cells were incubated with anti-CD3 for 24 hours in serum free culture media (Neuorbasal-A/B27) and activation of T cells was assessed using anti-CD69 post-incubation. Cells which were incubated for 24 hours without anti-CD3 in similar media was taken as a control. Both types of cells were treated in similar manner with anti-CD69. (A) Representative dot-plot showing activation of T cells compared with their non-activated phenotypes in serum free OSC culture media (Neuorbasal-A/B27). (B) Representative histogram showing up-regulation of CD69 marker in activated T cells. Red line represents activated T cells and green line represents non-activated T cells while blue line represents isotype control. The right hand shift of red graph represents the up-regulation of CD69 marker in comparison to green graph.

3.2.4 Lymphocytes do not induce toxicity to OSCs under normal culture conditions

Having established that lymphocyte preparations were viable and able to activate in serum-free OSC culture media, the present study has investigated the response of OSC to lymphocyte preparations under normal culture conditions to determine whether lymphocytes induce toxicity. OSCs were co-cultured with lymphocyte preparations in serum free OSC culture media for 18 hours before assessing cell viability in the slice using PI. The 18 hours time point was considered in the present study because the survival rate of lymphocytes after 18 hours was found to be inconsistent. The present data reveal that lymphocytes are not toxic to OSCs under normal culture conditions (Figure 3.7). Neurotoxicity was compared with slices treated with 300 μM KA for 1 hour and control slices, which were incubated in fresh culture media only. OSCs were also tested against lymphocytes containing either non-activated or activated T cells. Neither lymphocytes with non-activated T cells (2.1 ± 2.0 % of the 300 μM KA control; n = 17 slices from 6 animals; $p<0.001$) nor with activated T cells (0.3 ± 0.2 % of the 300 μM KA control; n = 9 slices from 4 animals; $p<0.001$) were harmful to OSCs under normal culture conditions.

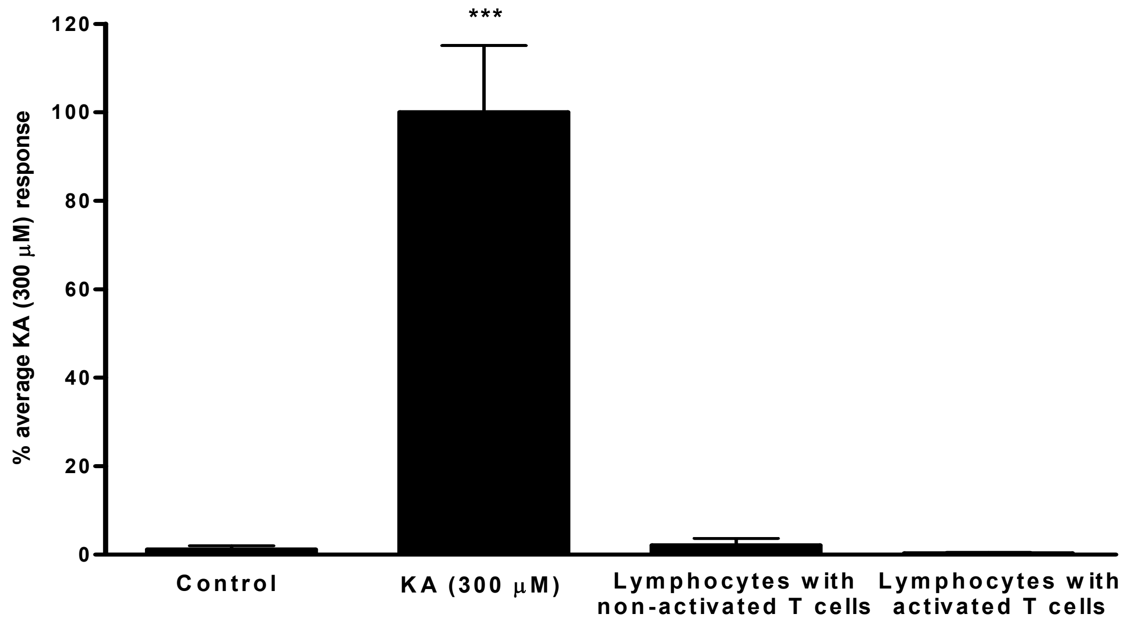


Figure 3.7 Lymphocyte preparations are not harmful to OSCs under normal culture condition.

Lymphocyte preparations containing either non-activated T cells or activated T cells after co-culturing with OSCs for 18 hours do not induce toxicity in OSCs. Toxicity was measured against slices treated with 300 μM KA for 1 hour and incubated in fresh culture media for 18 hours. All experiments were carried out in at least 4 separate animals. Data presented as mean ± SEM. ***p < 0.001 versus fresh culture media (control).

3.3 Discussion

It has been argued that the mode of action of cell death in aging and neurodegenerative diseases is similar to KA-induced cell death (Pellegrini-Giampietro *et al.*, 1997; Friedman, 1998; Arundine and Tymianski, 2003; Arundine and Tymianski, 2004; Wang *et al.*, 2005). In KA-induced cell death and neurodegenerative diseases, excitotoxicity and oxidative stress have been suggested to be the major cause of neurodegeneration (Sun *et al.*, 1992; Cheng and Sun, 1994; Arundine and Tymianski, 2003; Arundine and Tymianski, 2004). Moreover, it has been suggested that excitotoxicity mediated neuronal death is one of the key features in a number of CNS pathologies including stroke and PD (Choi *et al.*, 2005) and the major cause of neuronal death is mediated via oxidative stress (Gluck *et al.*, 2000). Furthermore, KA has been successfully used for inducing neuronal death in various studies to demonstrate neurodegeneration (Portera-Cailliau *et al.*, 1997; Gluck *et al.*, 2000; Greenwood and Bushell, 2010). Recently, neuroinflammation has been suggested to be one of the key factors in KA-induced neuronal death along with neurodegeneration (Järvelä *et al.*, 2010). Therefore, together with these findings and to determine whether the KA model can be used, I investigated the concentration response of KA-induced toxicity and validated the concentration of KA for the present experimental conditions. KA induced cell death in a concentration-dependent manner in the present experimental conditions. This result is similar to the previous observation in our laboratory which was performed in rat OSCs (Greenwood and Bushell, 2010), suggesting that both rat and murine OSCs are equally vulnerable to KA toxicity although the degree of cell death may vary. Consistent with previous studies (Koh *et al.*, 1990; Kato *et al.*, 1991; Sun *et al.*, 1992; Wang *et al.*, 2005), the present study shows that antagonists for NMDA and APMA/KA receptors block KA-induced cell death which suggests that it is mediated via ionotropic glutamatergic receptors (Figure 3.2).

The concentration-response curve has revealed that the EC50 of KA lies below 300 μM KA which suggests that the concentration of 300 μM KA is sufficient enough to induce

more than 50% of cell death but not maximum cell death. Therefore, 300 μM KA was considered to be an appropriate concentration for our experiments to test our hypothesis that lymphocytes are either pathogenic or neuroprotective under the present experimental conditions.

The present data also show that lymphocytes can be cultured in serum free OSC culture media as effectively as media containing serum (cRPMI). Normally, lymphocytes are cultured in serum containing media (Hickey *et al.*, 1991; Culshaw *et al.*, 2008) but lymphocytes were also reported to be cultured in serum free media using growth factors (Barnes and Sato, 1980; Herzberg and Smith, 1987). As OSCs were cultured in serum free culture media, it was important to determine whether T cells can be activated in serum free condition. In the present study, it has also been demonstrated that T cells can be activated in serum free OSC culture media as effectively as serum containing media using anti-CD3. In addition, I also showed that KA does not affect the survival of lymphocytes suggesting that KA is not toxic to lymphocytes. Supporting this finding, it has been shown that rodent lymphocytes lack AMPA/KA receptors (Boldyrev *et al.*, 2004).

The present data has revealed that under normal culture conditions, lymphocytes do not induce toxicity to OSCs. In support of this finding, Wolf *et al.* (2002) have also demonstrated that T cells when added beneath slices separated by the insert membrane were not toxic. Furthermore, I have also shown that the presence of either non-activated or activated T cells within lymphocyte preparations do not have any effect in the response of lymphocytes to OSC under normal culture condition. Consistent with this finding, several *in vivo* studies have shown that peripherally activated lymphocytes enter the healthy CNS via the BBB (Hickey, 1991; Hickey *et al.*, 1991; Engelhardt and Ransohoff, 2005) but do not initiate any inflammatory response till they recognise their cognate antigens and those lymphocytes which cannot recognise their cognate antigens exit from the CNS regularly (Hickey *et al.*, 1991; Becher *et al.*, 2006; Odoardi *et al.*, 2007; Bartholomaus *et al.*, 2009). There is also evidence for infiltration of non-activated

lymphocytes into the CNS via the blood-CSF barrier and are suggested to be a normal recirculating lymphocyte pool of the CSF (Seabrook *et al.*, 1998). When these findings are taken together, it can be suggested that in normal CNS, lymphocyte preparations containing either non-activated or activated T cells do not initiate inflammatory responses..

In summary, the present finding shows that KA can be used to induce cell death in OSCs to investigate the response of lymphocytes in neurodegeneration. Further, I also demonstrated that lymphocytes are viable in serum-free OSC culture media and can be activated in serum-free OSC culture media. The present study has also revealed that lymphocytes are not affected by KA. In addition, I showed that lymphocytes do not induce toxicity to OSCs under normal culture conditions. These findings indicate that the present experimental setups can be used to test a hypothesis that "Lymphocytes play a crucial role in the CNS during neurodegenerative diseases".

4 Role of lymphocytes in two different models of neurodegenerative disease

As the main aim of the thesis was to determine the role of lymphocytes in neurodegenerative diseases, I used two models of neurodegenerative diseases: KA model of neurodegeneration and OGD model for an *in vitro* model of stroke. In chapter 3, I showed that lymphocytes do not induce toxicity to OSCs under normal culture conditions. Here, in this chapter, I am presenting evidence for the response of lymphocytes in these two different neurodegenerative disease models. This chapter will give a brief background on the role of lymphocytes in neurodegenerative diseases followed by results and discussion using the KA and OGD model.

4.1 Introduction

As highlighted in the introduction chapter, neurodegenerative diseases have huge impact on the global burden of diseases as well as on healthcare cost (Cruz *et al.*, 2006; Nutt, 2011). However, the pathology of these diseases is still poorly understood. Generally, these diseases are characterised by a progressive loss of neurons from the CNS with a deficit in the affected part or region. Although various conditions including ageing (Collier *et al.*, 2011; Tollervey *et al.*, 2011), stress and trauma (Bramlett and Dietrich, 2004; Sugama, 2009) and genetic disposition (Butterfield *et al.*, 2000; Elbaz *et al.*, 2007) may induced cell death during neurodegeneration, recently, studies have shown a strong relationship between neurodegeneration and inflammation (Campbell, 2004; Lucas *et al.*, 2006; DeLegge and Smoke, 2008; Amor *et al.*, 2010). During neurodegenerative diseases, either peripheral immune cells, including lymphocytes, initiate inflammation in the CNS (Hickey and Kimura, 1988; Neumann, 2001; Kivisäkk *et al.*, 2009) or CNS-resident immune competent cells such as microglia, astrocytes, neurons and oligodendrocytes release inflammatory mediators to recruit more peripheral immune cells into the CNS (Block and Hong, 2005; Lucas *et al.*, 2006) leading to the CNS inflammation. There is also evidence that meninges release pro-inflammatory cytokines

including TNF-α, IL-1 and IL-6 in response to inflammatory stimuli (Wieseler-Frank *et al.*, 2007) and meningeal antigen presenting cells can activate T cells to initiate inflammatory responses in neurodegenerative diseases such as EAE (Kivisäkk *et al.*, 2009). As described in the introduction chapter, several studies have demonstrated the infiltration of lymphocytes in neurodegenerative diseases (McFarland and Martin, 2007; Rezai-Zadeh *et al.*, 2009; Chung *et al.*, 2010; Saresella *et al.*, 2011) but their precise role during neurodegeneration is still a matter of debate. Various studies have suggested that lymphocytes contribute to exacerbation of neurodegeneration during neurodegenerative diseases (Fee *et al.*, 2003; Appel *et al.*, 2009; Brochard *et al.*, 2009; Huang *et al.*, 2009). In contrast, other studies have argued for a beneficial role of lymphocytes during neurodegenerative diseases (Moalem *et al.*, 1999; Moalem *et al.*, 2000; Schwartz, 2005; Beers *et al.*, 2008).

I have also investigated the role of lymphocytes in stroke using an *in vitro* model. Stroke is one of the major causes of morbidity and mortality in industrialised countries (Yilmaz and Granger 2010) yet there is no effective therapy for this problem. Recent evidence links inflammatory status and the risk of stroke (Wang *et al.*, 2007; McColl *et al.*, 2009; Denes *et al.*, 2010) as stroke itself can trigger the inflammatory response by local cellular activation and release of various inflammation-related mediators including cytokines, chemokines, proteases, reactive oxygen species and upregulation of adhesion molecules (Wang *et al.*, 2007) which lead to the recruitment of peripheral immune cells including lymphocytes into the brain (Rosenberg, 1999; Danton and Dietrich, 2003; Yilmaz *et al.*, 2006; Wang *et al.*, 2007). Both *in vivo* (Ortolano *et al.*, 2010; Fumagalli *et al.*, 2011) as well as *in vitro* (Schroeter *et al.*, 1994; Jander *et al.*, 1995) studies have demonstrated the infiltration of lymphocytes into the stroke brain. However, these studies were lacking on demonstrating the precise role of T cells during stroke. Although, researches have argued on detrimental (Yilmaz *et al.*, 2006; Hurn *et al.*, 2007; Liesz *et al.*, 2011) or protective (Frenkel *et al.*, 2005; Planas and Chamorro, 2009; Ren *et al.*, 2011) role of either T cells or B cells, still more researches seemed required to know the precise role of these cells during stroke. Most of these *in vivo* studies were

targeted to specific cell types such as either T cells or B cells and outcomes of these models can also be largely affected by peripheral inflammatory responses (Spencer *et al.*, 2007) as well as other interacting factors including anaesthesia, temperature and cerebrovascular changes (Dong *et al.*, 1988). So far, however, there has been little discussion about the role of lymphocytes in ischemic cell death during stroke.

Therefore, in an attempt to address the precise role of lymphocytes in neurodegenerative diseases including stroke, the present study has investigated the role of lymphocytes in neurodegeneration using the KA and OGD model. Moreover, the current study has used an *in vitro* stroke model to determine the role of lymphocytes in OSCs after subjected to OGD as this model has been suggested to be useful to differentiate neuronal death by ischaemia rather than from cerebrovascular system (Dong *et al.*, 1988).

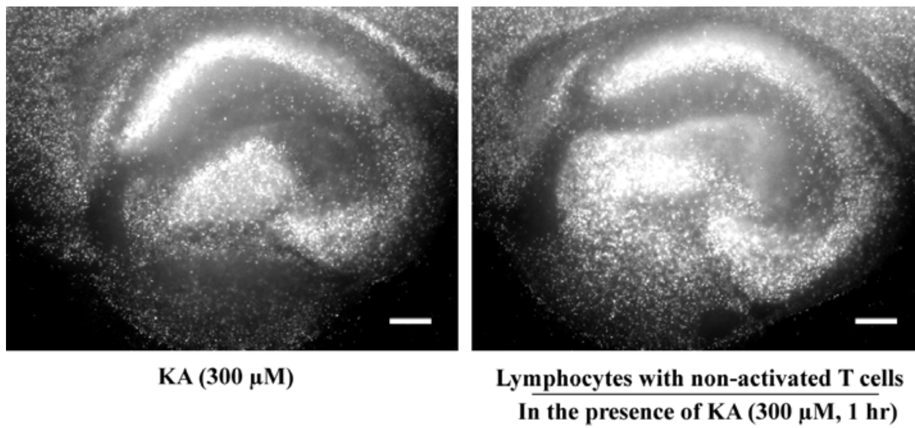
4.2 Results

4.2.1 Lymphocyte preparations containing either non-activated T cells or activated T cells are neuroprotective against KA-induced cell death

After establishing in Chapter 3 that lymphocytes either in the non-activated or activated state do not induce toxicity to OSCs under normal culture conditions, I investigated the role of lymphocytes against KA-induced neurotoxicity in OSCs. First, I investigated whether lymphocytes in the presence of KA can modulate KA-induced toxicity. For this purpose, slices were co-cultured with lymphocyte preparations containing non-activated T cells in the presence of KA (300 μM) for 1 hour and incubated in fresh culture media with PI (2 μM) for 18 hours. Quantification of PI uptake showed 111.1 ± 13.7 % of cell death (n = 15 slices from 8 animals; $p > 0.05$ versus 300 μM KA; Figure 4.1) which suggest that lymphocytes do not modulate KA-induced toxicity. Furthermore, as several studies have shown either neurotoxic (Brochard *et al.*, 2009; Huang *et al.*, 2009) or neuroprotective (Schwartz, 2005; Beers *et al.*, 2008) role of activated T cells, T cells within the lymphocyte preparations were activated using anti-CD3 (see section 2.2.2.3) to investigate their role under the present experimental conditions. Similar to the previous experimental condition, after treating slices with KA for 1 hour in the presence of lymphocyte preparation containing activated T cells, slices were incubated in fresh culture media with PI for 18 hours and quantified cell death. Lymphocyte preparations containing activated T cells exhibited 113.2 ± 12.4 % of cell death (n = 14 slices from 6 animals; $p > 0.05$ versus KA 300 μM) when compared with 300 μM KA and no significant differences were found in the level of response between lymphocyte preparations containing non-activated T cells and lymphocyte preparations containing activated T cells. This suggests that activation of T cells within the lymphocyte preparations does not modulate KA-induced cell death.

To investigate whether a longer incubation period with lymphocyte preparations can modulate KA-induced toxicity, I therefore modified the model such that slices were treated with KA for 1 hour and co-cultured with lymphocyte preparations containing non-activated T cells for 18 hours. As shown in Figure 4.2, lymphocyte preparations containing non-activated T cells significantly reduced KA-induced cell death (72.1 ± 5.1 % of the 300 μM KA control; n = 48 slices from 18 animals; $p < 0.01$) which suggests that lymphocytes are neuroprotective. In addition, as the pathogenic response of activated T cells has been demonstrated by various studies (Appel, 2009; Brochard *et al.*, 2009; Huang *et al.*, 2009), I also investigated whether the presence of activated T cells within the lymphocyte preparations have an effect on the observed neuroprotection exhibited by the lymphocyte preparations containing non-activated T cells. Having established in Figure 3.6 that anti-CD3 activated T cells within the lymphocyte preparations do not induce toxicity and lymphocyte preparations containing non-activated T cells are neuroprotective, I treated slices with KA for 1 hour and incubated with lymphocyte preparations containing activated T cells for 18 hours. Lymphocyte preparations containing activated T cells reduced neuronal death against KA-induced toxicity (64.6 ± 5.0 % of the 300 μM KA control; n = 50 slices from 17 animals; $p < 0.001$). Moreover, no significant differences were found in the level of neuroprotection between lymphocyte preparations containing non-activated T cells and lymphocyte preparations containing activated T cells. Together, these data suggest that cells present in the lymphocyte preparations were associated with neuroprotection under the present experimental conditions but their neuroprotection is time dependent. However, as these lymphocyte preparations were generated from whole lymph nodes, it was next important to determine the types of cells associated with this neuroprotection.

(A)



(B)

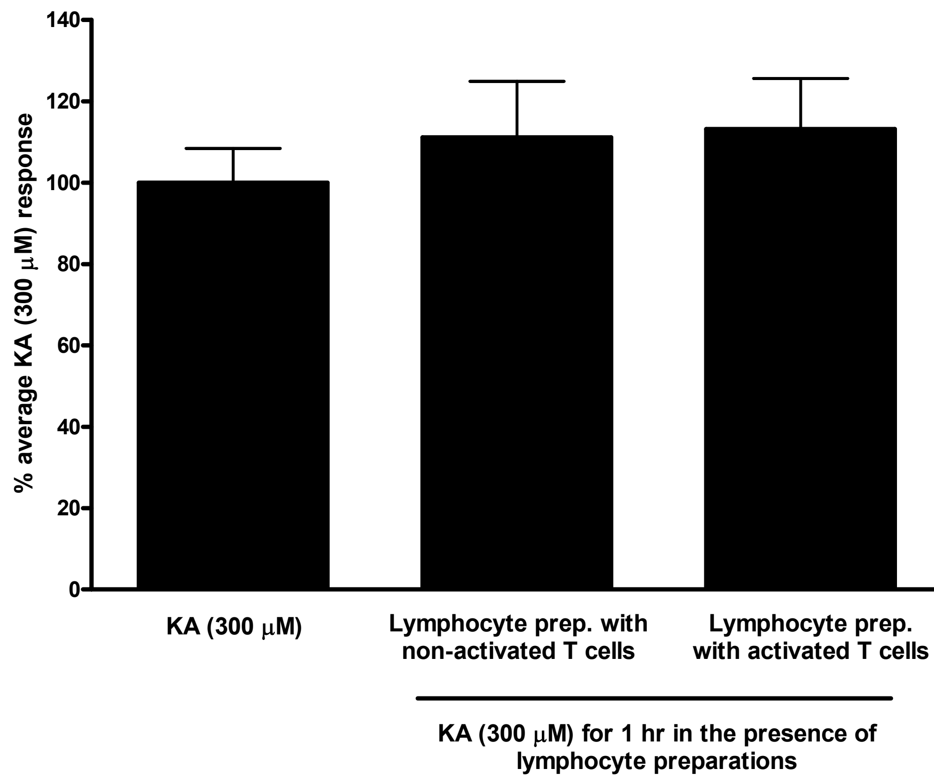


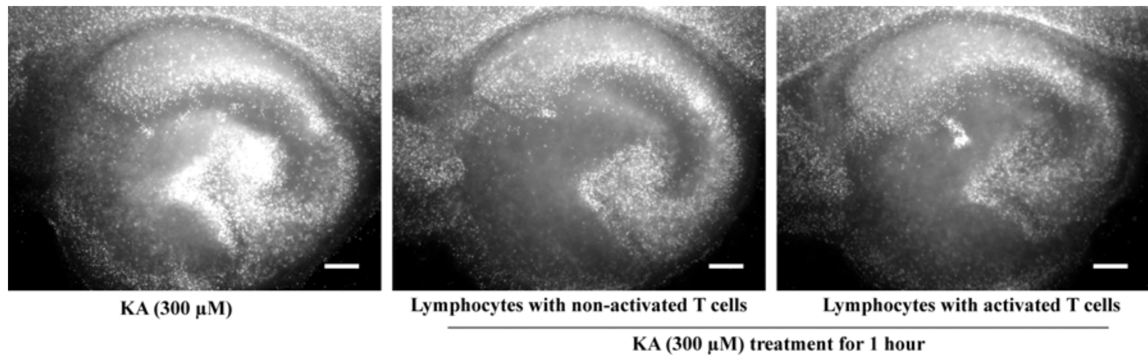
Figure 4.1 Lymphocytes do not alter KA-induced cell death when co-cultured for 1 hour in the presence of KA.

Continued…

Figure 4.1 Lymphocytes do not alter KA-induced cell death when co-cultured for 1 hour in the presence of KA.

Following culturing OSCs for 13-15 days in culture media, they were treated with KA (300 μM, 1 hr) in the presence of $1X10^6$ lymphocytes beneath the insert membrane and incubated in fresh culture media for 18 hours in the presence of PI (2 μM). The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices cultured with or without lymphocyte preparations either containing non-activated or activated T cells in the presence of KA which were then incubated in fresh culture media for 18 hours. (B) Bar chart illustrating the quantification of cell death after slice treated with KA (300 μM) for 1 hour in the presence of lymphocytes and transferred to fresh media for recovery. All experiments were carried out in at least 6 separate animal preparations. Data presented as mean ± SEM. Scale bar: 200 μm.

(A)



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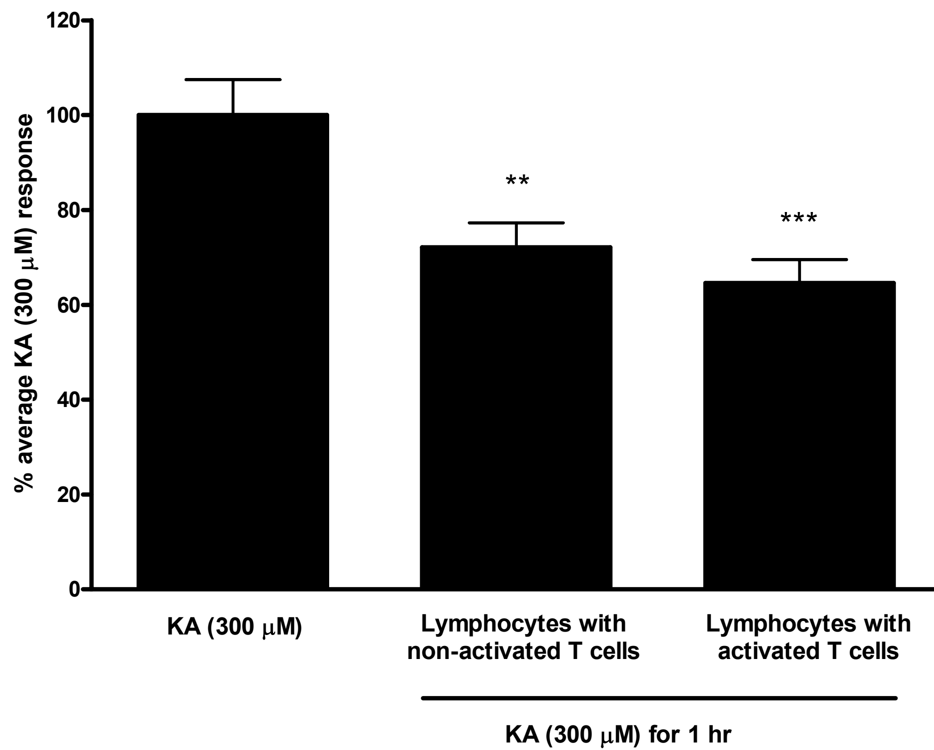


Figure 4.2 Lymphocytes are neuroprotective against KA-induced neurotoxicity.

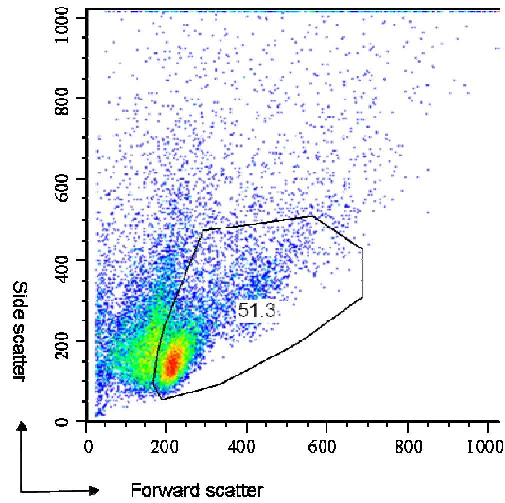
Continued…

Figure 4.2 Lymphocytes are neuroprotective against KA-induced neurotoxicity. Slices were cultured for 13-15 days in culture media in prior to experiments. Slices were treated with KA (300 µM, 1 hr) and incubated with $1X10^6$ lymphocytes beneath the insert membrane for 18 hours in the presence of PI (2 µM). The PI images were visualised using fluorescence microscope. (A) Representative PI fluorescence images showing cell death in slices treated with KA and incubated with lymphocyte preparations containing either non-activated or activated T cells for 18 hours. (B) Bar chart demonstrating the reduction of cell death in slices co-cultured with lymphocyte preparations containing either non-activated T cells or activated T cells. All experiments were carried out in at least 15 separate animal preparations. Data presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ versus KA (300 µM). Scale bar: 200 µm.

4.2.2 CD4+ and CD8+ lymphocytes dominant in lymphocyte preparation

Having established that lymphocyte preparations were neuroprotective against KA-induced neurotoxicity, I investigated which specific cell population within lymphocyte preparations contribute to the observed neuroprotection. The neuroprotective role of T cells has been demonstrated by several studies (Wolf *et al.*, 2002; Schwartz, 2005; Beers *et al.*, 2008). Therefore, flow cytometry was carried out on lymphocyte preparations to investigate T cell populations using anti-CD4 and anti-CD8 antibodies. Flow cytometry showed that more than 60% of the cells in the lymph nodes (LN) were $CD4^{+/}CD8^{+}$ lymphocytes (Figure 4.3B). The T cell population within lymphocyte preparations was found to be 68.4 ± 1.8 % (n = 16 samples from 4 animals) of the total lymphocyte population which was identified by forward scatter (FSC) versus side scatter (SSC) gating (Figure 4.3A).

(A)



(B)

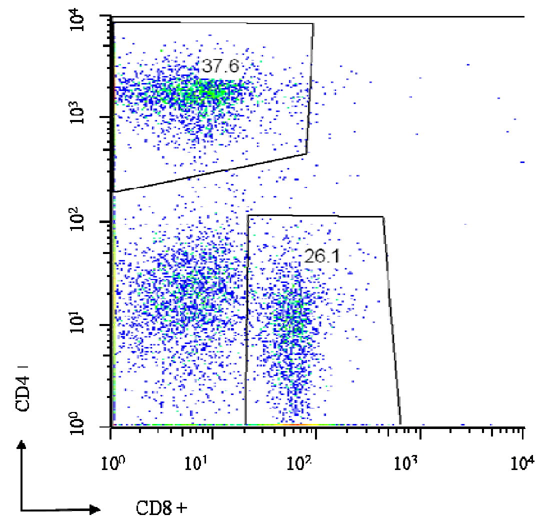


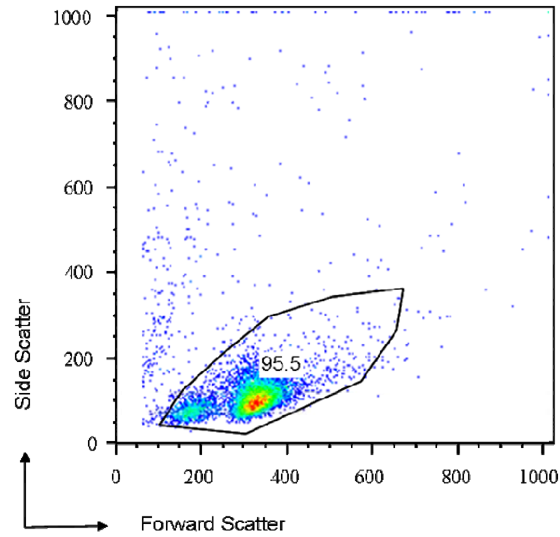
Figure 4.3 T cells are the dominant cell population within lymphocyte preparations. The percentage of T cells was determined by labelling them using anti-CD4 and anti-CD8 and representative dot-plot showing (A) lymphocytes were gated against FSC and SSC. (B) top left hand population is $CD4^+$ lymphocytes and right hand population represents $CD8^+$ lymphocytes whereas cells in left hand population near axis represents all non $CD4^{+/}CD8^+$ lymphocytes. The combination of $CD4^+$ cells and $CD8^+$ cells represents the total number of T cells within lymphocyte preparations which account for more than 60% of lymphocyte population.

4.2.3 T cells exhibit neuroprotection against KA-induced cell death

Having demonstrated that over 60% of the cells in lymphocyte preparation were CD4 or CD8 T cells, I wanted to determine whether these cells were responsible for the neuroprotection as seen in Fig 4.2. T cells were therefore purified using a negative selection strategy in magnetic separation. Following isolation, the purity of T cells was assessed using CD3 as a marker for T cells and found to be 98.5 ± 0.4 % ($n = 6$ samples from 6 animals) of the purified cell population (Figure 4.4).

After determining the purity of T cells after isolation, I investigated whether purified T cells were contributing neuroprotection against KA-induced cell death as shown in Figure 4.2. Slices were treated with KA (300 μM, 1 hour) and incubated with media containing purified non-activated T cells for 18 hours in the presence of PI (2 μM). The purified T cells reduced cell death significantly (67.8 ± 5.9 % of the 300 μM KA control; $n = 16$ slices from 9 animals; $p < 0.001$; Figure 4.5) against KA-induced neurotoxicity. Again, to determine whether activation of purified T cells can alter the observed neuroprotection, I also investigated the role of purified activated T cells which were activated by anti-CD3/anti-CD28 as the neuroprotection of activated T cells has been shown by several studies (Moalem *et al.*, 2000; Beers *et al.*, 2008). Following treatment with KA, slices were incubated with purified activated T cells for 18 hours and assessed PI uptake. As similar to the purified non-activated T cells, the purified activated T cells also significantly reduced KA-induced neurotoxicity (76.2 ± 4.2 % of the 300 μM KA control; $n = 7$ slices from 5 animals; $p < 0.05$). In addition, there were no significant differences in the neuroprotective response between purified non-activated T cells and purified activated T cells. These findings suggest that the neuroprotection exhibited by T cells is independent of the activation state of T cells.

(A)



(B)

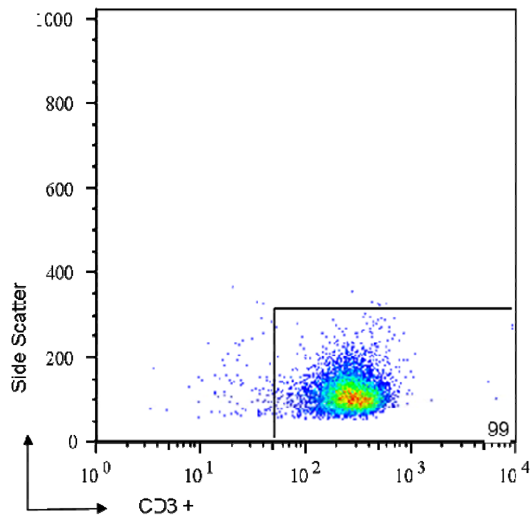
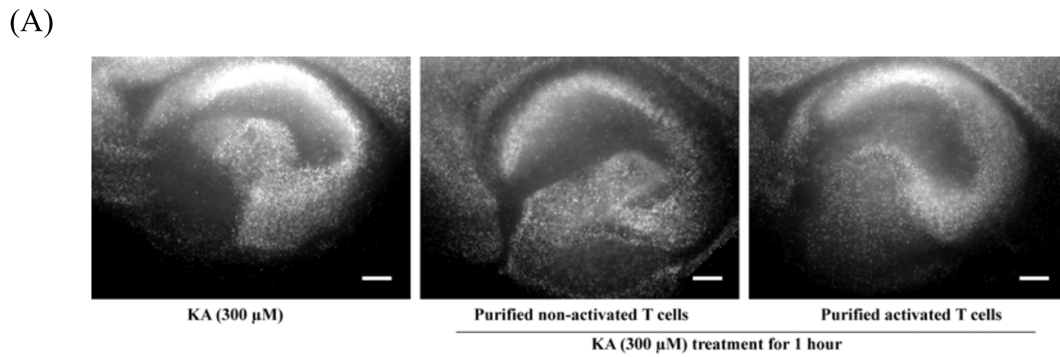


Figure 4.4 Purified cells from lymphocyte preparations are T cells.

T cells were identified using anti-CD3 and representative dot-plot showing (A) gating of lymphocytes which were determined against side scatter and forward scatter. (B) right hand population showing T cell population within the purified cells.

(A)



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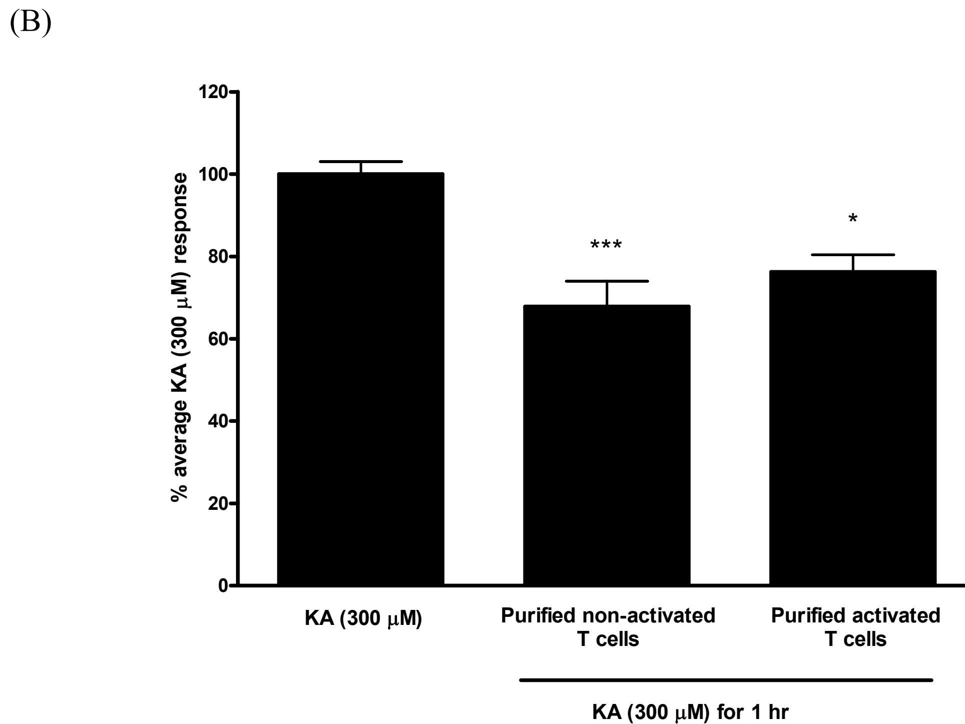


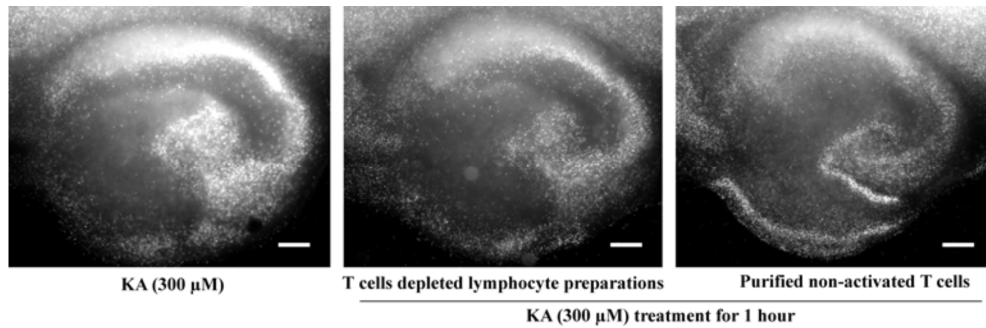
Figure 4.5 The neuroprotection against KA-induced cell death is independent of the activation of T cells.

Slices were treated as described in Figure 4.2. (A) Representative PI fluorescence images of slices treated with KA (300 µM) for 1 hour and co-cultured with either purified non-activated T cells or purified activated T cells for 18 hours. (B) Bar charts showing the response of purified non-activated T cells and purified activated T cells against KA-induced neurotoxicity. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ versus KA 300 µM. Scale bar: 200 µm.

4.2.4 Non-T cells also exhibit neuroprotection against KA-induced cell death

Having demonstrated a neuroprotective role of purified T cells against KA-induced cell death, I wanted to find out whether T cells alone were contributing to neuroprotection under the present experimental conditions. I therefore investigated the role of non-T cells against KA-induced neurotoxicity to determine whether these cells also contribute to the neuroprotection as seen in Figure 4.2. Following the separation of T cells from lymphocyte preparations, non-T cells were also collected. OSCs were treated with KA (300 μM, 1 hour) and incubated with either non-T cells or purified non-activated T cells for 18 hours. Interestingly, non-T cells were also neuroprotective against KA-induced cell death (74.3 ± 5.0 % of the 300 μM KA control; n = 16 slices from 9 animals; $p < 0.05$; Figure 4.6) as similar to the purified non-activated T cells (78.2 ± 4.4 % of the 300 μM KA control; n = 20 slices from 10 animals; $p < 0.05$) suggesting that non-T cells also contribute in the observed neuroprotection as seen in Figure 4.2. As a control experiment, slices were treated with KA for 1 hour and incubated with lymphocyte preparations for 18 hours. Consistent with the previous result (Figure 4.2), lymphocyte preparations showed a significant reduction in KA-induced cell death (42.4 ± 5.8 % of the 300 μM KA control; n = 8 slices from 6 animals; $p < 0.001$). Next, I determined types of cells present in the non-T cell preparations.

(A)



(B)

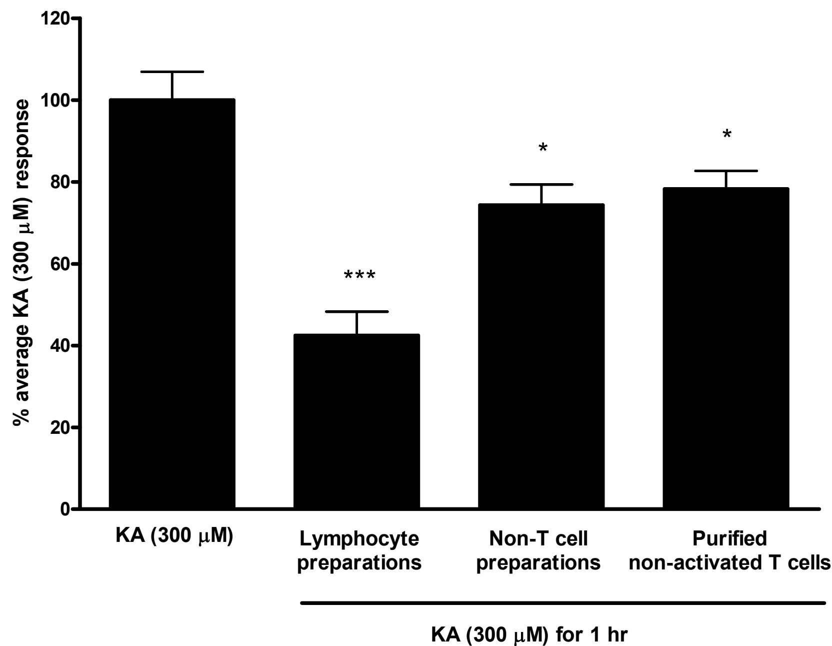


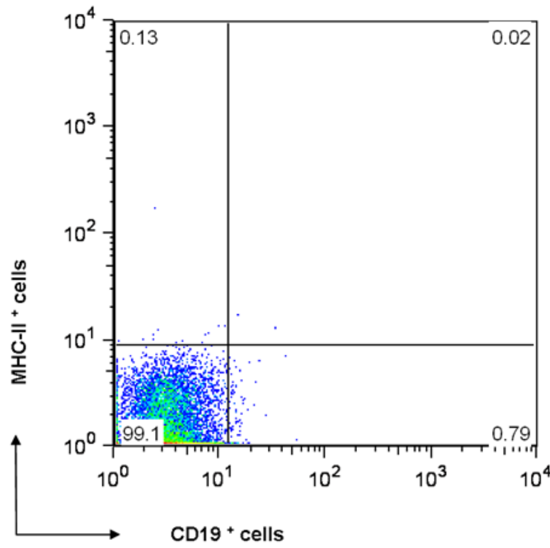
Figure 4.6 T cell depleted lymphocyte preparations (non-T cells) show a similar level of neuroprotection as purified non-activated T cells.

OSCs were treated as described in Figure 4.2. (A) Representative PI fluorescence images of slices treated with KA (300 μM) for 1 hour and co-cultured with either non-T cells or purified non-activated T cells for 18 hours. (B) Summary showing the response of lymphocyte preparations, non-T cells and purified non-activated T cells against KA-induced neurotoxicity. All experiments were carried out in at least 6 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ versus KA 300 μM. Scale bar: 200 μm.

4.2.5 B cells are the major cell population in non-T cell preparations

Following the neuroprotection observed against KA-induced cell death by non-T cells, I investigated the major cell population in non-T cells. Several studies have reported a possible neuroprotective role of B cells in neurodegenerative diseases (Wolf *et al.*, 1996; Fillatreau *et al.*, 2002; Kala *et al.*, 2010; Berer *et al.*, 2011). Therefore, flow cytometry was carried out on non-T cell preparations to determine the population of B cells within non-T cells using anti-MHC-II and anti-CD19 antibodies. CD19 is a B cell specific glycoprotein forming a B cell receptor (BCR) complex (Stamenkovic and Seed, 1988; Chalupny *et al.*, 1993) and can be used as a marker to identify B cells. The flow cytometric analysis revealed that double positive MHC-II/CD19 cells account for 69.8 ± 2.1 % (n = 4 samples from 4 animals) of total non-T cell preparations (Figure 4.7 B) which suggested that the majority of non-T cells were B cells.

(A) (B)



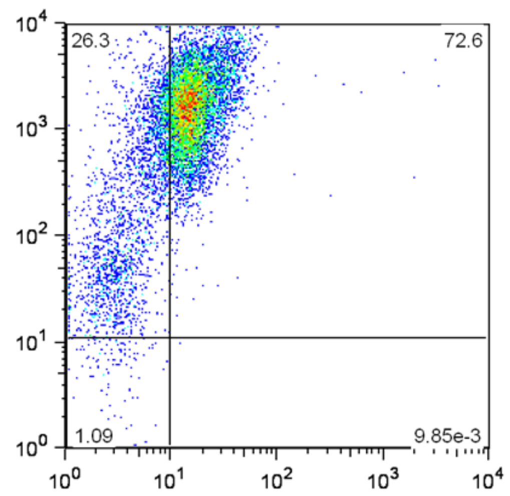
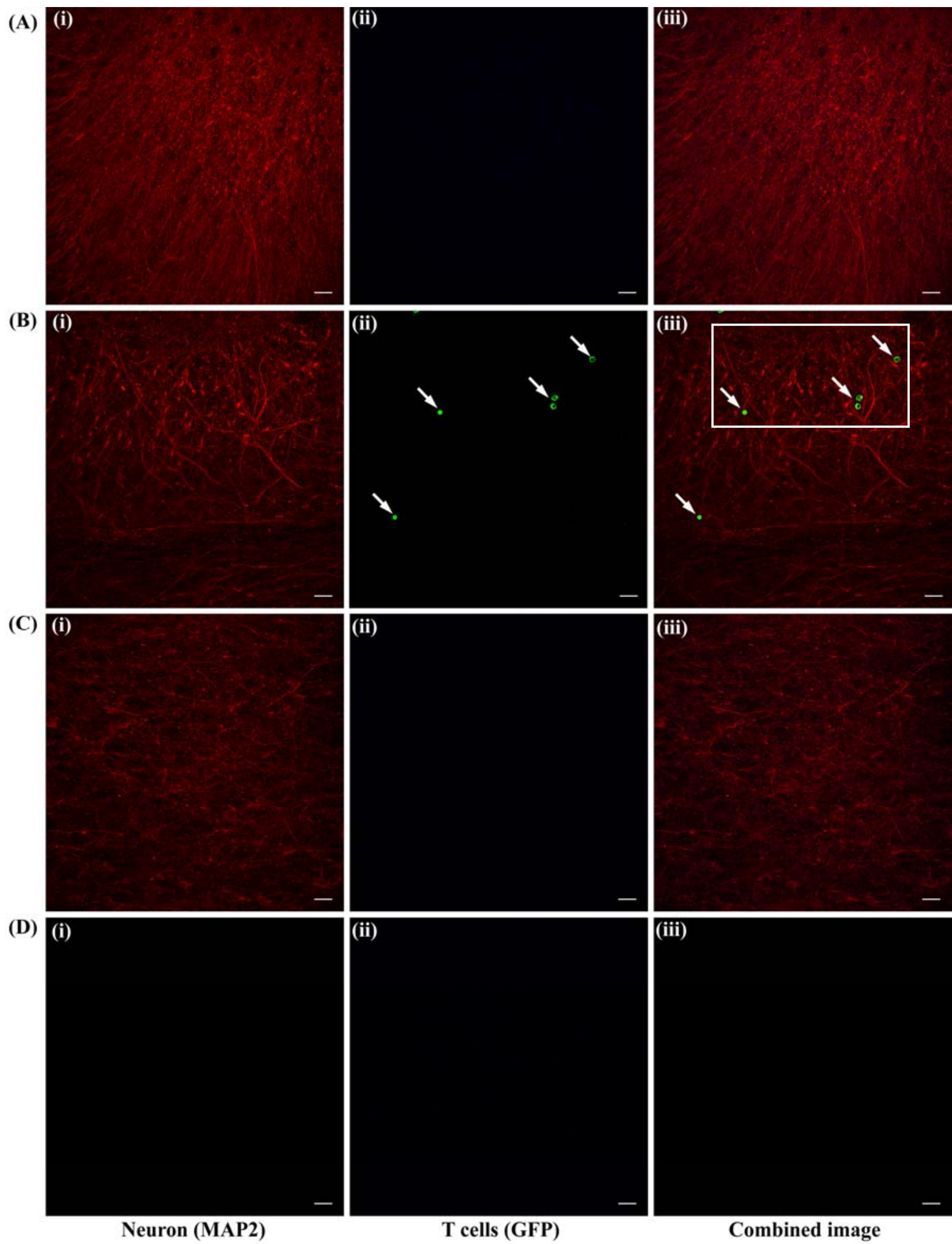


Figure 4.7 B cells are the dominant cells in non-T cells preparations.

T cell depleted cells were labelled using anti-MHC-II and anti-CD19 to determine B cell population. Representative dot-plot showing (A) left hand population of cell near to axis represents unstained lymphocytes which were negative for CD19 and MHC-II. (B) the double positive MHC-II/CD19 cells in top right side of dot plot represents B cells.

4.2.6 Neuroprotection mediated by lymphocytes is contact-independent

After establishing that lymphocyte preparations along with purified T cells and non-T cells exhibit neuroprotection, I also investigated whether the observed neuroprotection resulted from the direct interaction of lymphocytes and cells within the OSCs. GFP expressing T cells were used to determine whether lymphocytes can migrate through the insert membrane on which OSCs were cultured (see section 2.2.1.2) and interact with cells within the OSCs. Immunohistochemical analysis of OSCs showed that there was no infiltration of T cells into OSCs through the insert membrane when GFP expressing T cells were added beneath the insert membrane (Figure 4.8). As a positive control, GFP expressing T cells were added on the top of slice and under these conditions T cells were clearly evident within the slice (Figure 4.8 B and 4.8 E). To investigate whether migration of T cells can be induced by any toxic insult, OSCs were treated with KA (300 μM, 1 hour) and incubated in media containing lymphocytes for 18 hours. Immunohistochemistry of these OSCs also demonstrated that there was no migration of T cells through the insert membrane (Figure 4.8 C). For a negative control, only secondary antibodies were added to slices with lymphocyte preparations containing GFP expressing T cells on the top without primary antibodies treatment to exclude background staining (Figure 4.8 D).



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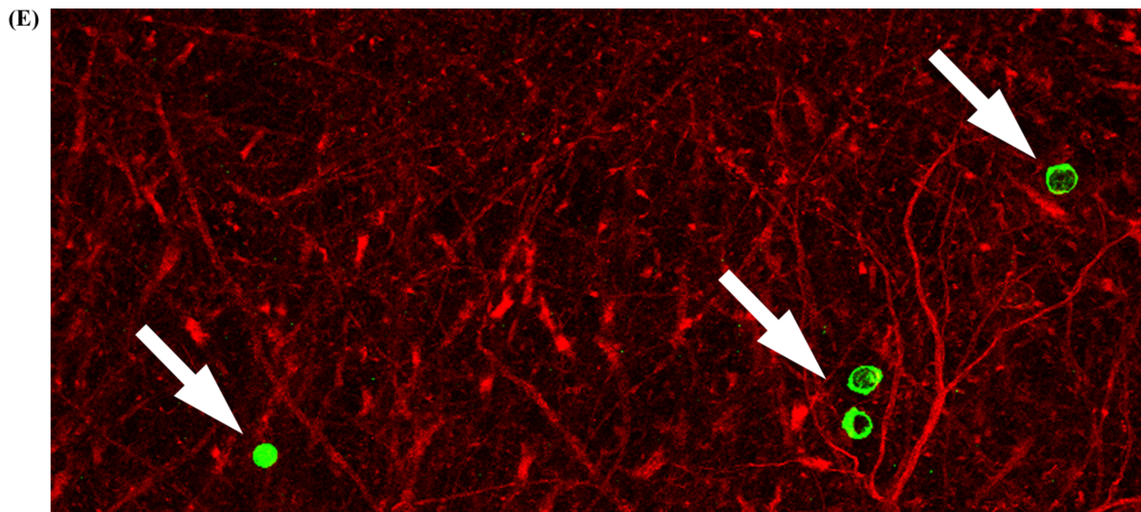


Figure 4.8 Lymphocyte-mediated neuroprotection is contact independent.
Representative confocal images showing neurons, microglia and GFP-expressed T cells after lymphocytes were added either (A) beneath insert membrane supporting the slice (B) on top of slice or (C) beneath the insert membrane supporting a KA-treated slice. After 18 hours of co-culture, slices were stained for (i) neurons labelled with anti-MAP2-Alexa Fluor 555 (A31570; red) (ii) T cells identified with anti-GFP-Alexa Fluor 488 (A21206; green) and (iii) images of neurons and T cells overlaid. (D) Representative confocal image for negative control slice where only secondary antibodies were added. (E) Magnified image from B (iii) showing T cells localised in the slice. Arrow heads showing T cells in all images. Neurons are shown in red and T cells in green. Scale bar: 200 μm.

4.2.7 Lymphocyte-mediated neuroprotection is mediated by soluble mediators secreted from lymphocytes

Having demonstrated an important role for lymphocytes in neuroprotection (Figure 4.2), but that this effect was contact-independent (Figure 4.8), I next considered whether conditioned media from lymphocyte preparations might be neuroprotective. As the present experimental conditions have various sources of soluble mediators including lymphocytes, the slice itself or a combination, I therefore, used three different types of conditioned media to determine if any were able to transfer the neuroprotection seen by lymphocyte preparations:

Conditioned media-I (lymphocytes exposed to OSCs)
Conditioned media-II (lymphocytes alone)
Conditioned media-III (KA treated slice)

To confirm that the observed neuroprotection is associated with lymphocytes but not with other cells, I used conditioned media from rat primary culture which was referred to as conditioned media-IV.

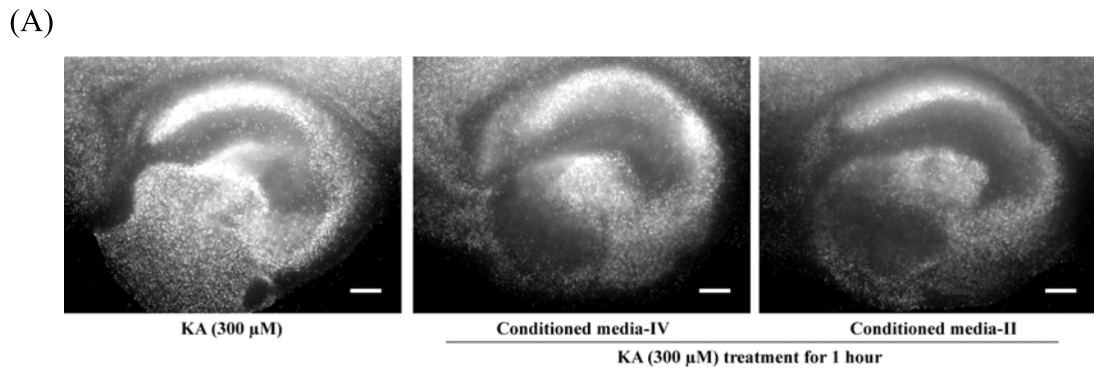
OSCs were treated with KA for 1 hour and subsequently transferred into the appropriate conditioned media (as described above) for 18 hours, before analysing neurodegeneration with PI staining. When KA-treated OSCs were incubated in conditioned media-I, neuroprotection against KA-induced neurotoxicity was evident (58.1 ± 7.2 % of the 300 μM KA control; $n = 11$ slices from 5 animals; $p < 0.001$; Figure 4.9). This observation demonstrates that conditioned media from lymphocytes exposed to OSCs is neuroprotective. To confirm conditioned media from lymphocytes was neuroprotective, KA-treated OSCs were incubated in conditioned media-II and PI uptake quantified. Neuroprotection against KA-induced neurotoxicity was observed (54.5 ± 3.6 % of the 300 μM KA control; $n = 10$ slices from 5 animals; $p < 0.001$; Figure 4.9) with no significant differences in the level of neuroprotection between conditioned media-I

and conditioned media-II. This suggests that lymphocytes alone are sufficient to give neuroprotection against KA-induced cell death. As a clear benefit of conditioned media-I in comparison to conditioned media-II could not be identified in the present study, conditioned media-II was used for later studies.

Next, to confirm whether the conditioned media specifically from lymphocytes is neuroprotective, I used conditioned media-III which was obtained from OSCs treated with KA 300 μM for 1 hour (see section 2.2.6.1.3) and conditioned media-IV which was obtained from supernatant of rat primary hippocampal cultures (see section 2.2.6.1.4). OSCs were treated with KA and incubated in conditioned media-III. Conditioned media-III failed to exhibit neuroprotection (92.5 ± 5.7 % of the 300 μM KA control; n = 9 slices from 6 animals; $p > 0.05$ versus KA 300 μM). Consistent with the finding for conditioned media-III, when OSCs treated with KA (300 μM, 1 hour) were incubated in conditioned media-IV, it failed to block KA-induced neurotoxicity (90.0 ± 3.8 % of the 300 μM KA control; n = 9 slices from 5 animals; $p > 0.05$ versus KA 300 μM). This finding implies that lymphocytes are responsible for the observed neuroprotection and that this is mediated by soluble factors, but independent of OSCs.

To investigate whether soluble mediators present in conditioned media-II are proteinaceous, conditioned media-II was heated which will denature proteins present in the media as described previously (see section 2.2.6.1.5). When OSCs were treated with KA and incubated in heated conditioned media-II, the observed neuroprotection was abolished (94.2 ± 8.2 % of control; n = 6 slices from 5 animals; $p > 0.05$ versus KA 300 μM Figure 4.10) suggesting that lymphocytes release soluble mediators are proteins which are responsible for the neuroprotection.

(A)



(B)

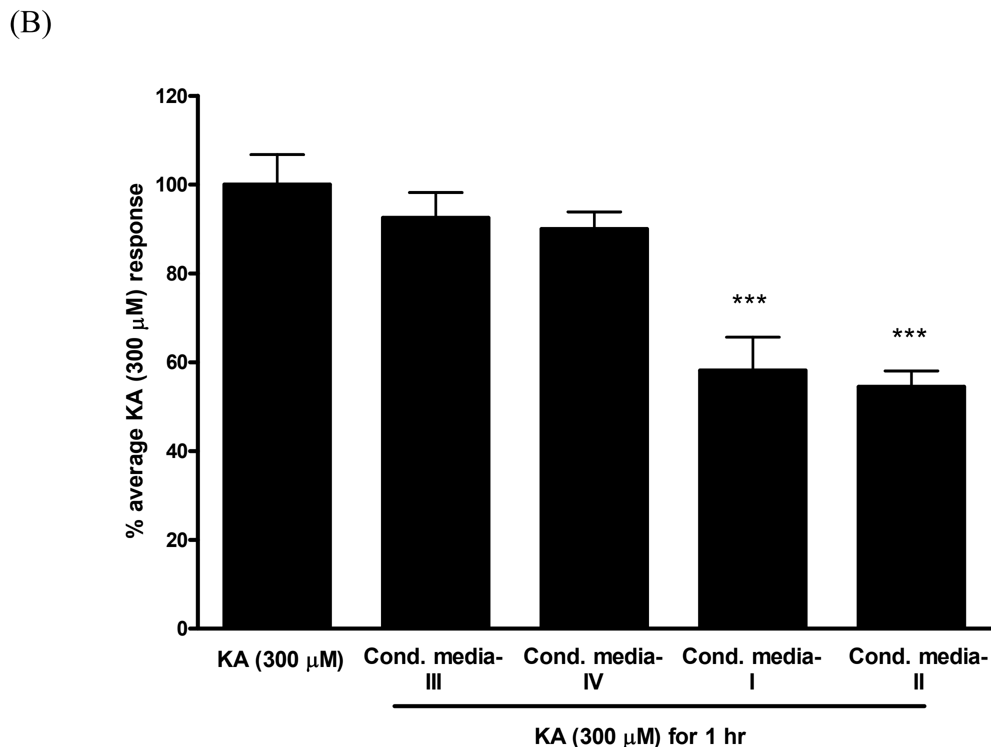


Figure 4.9 Soluble mediators released by lymphocytes are neuroprotective against KA-induced cell death.

OSCs were treated as described in Figure 4.2. (A) Representative PI fluorescence images of slices treated with KA (300 μM) for 1 hour and incubated in either conditioned media-I or conditioned media-II or conditioned media-IV for 18 hours. (B) Bar chart demonstrating the neuroprotection exhibited by conditioned media-I and II but not by conditioned media-III and IV. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. ***$p < 0.001$ versus KA 300 μM. Scale bar: 200 μm.

(A)

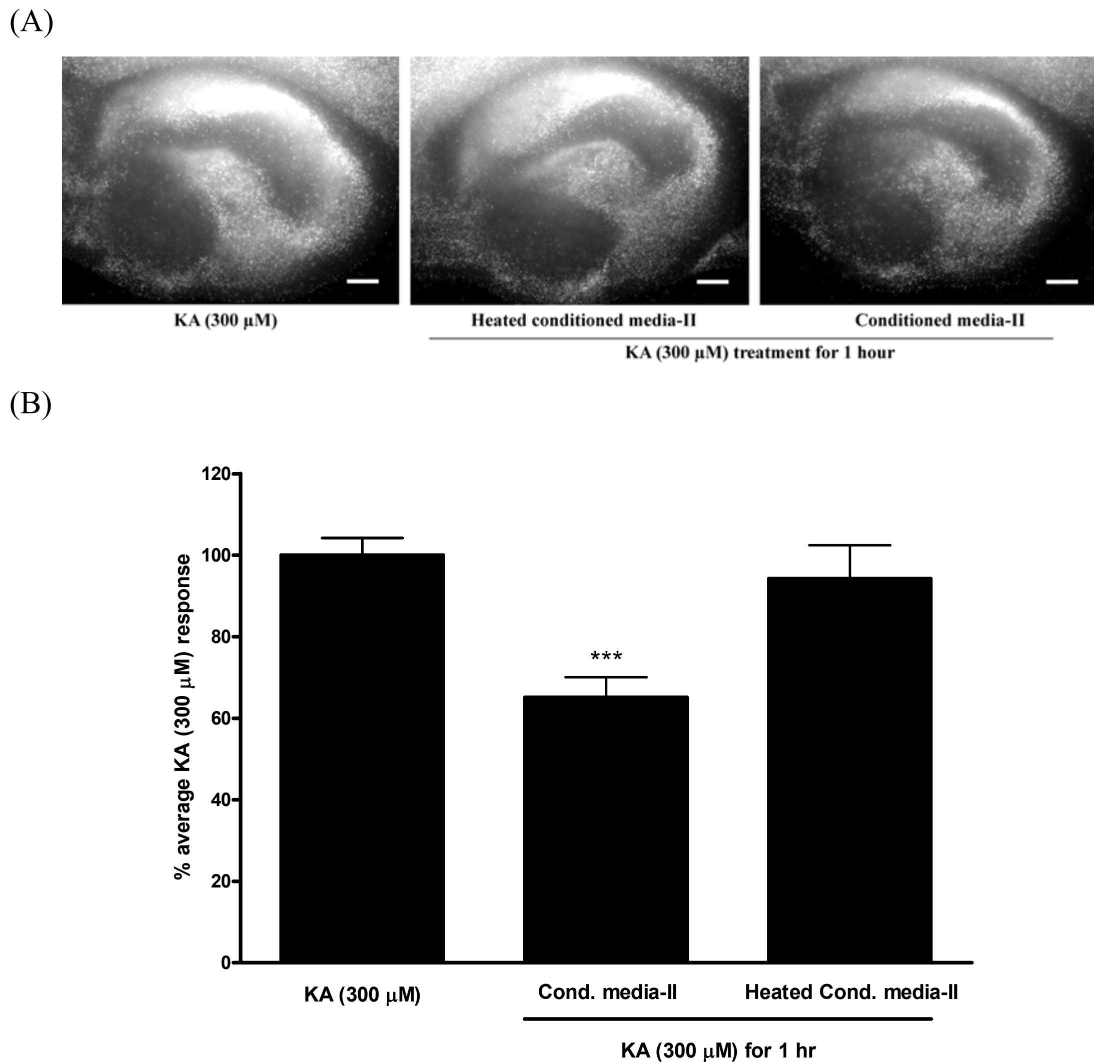


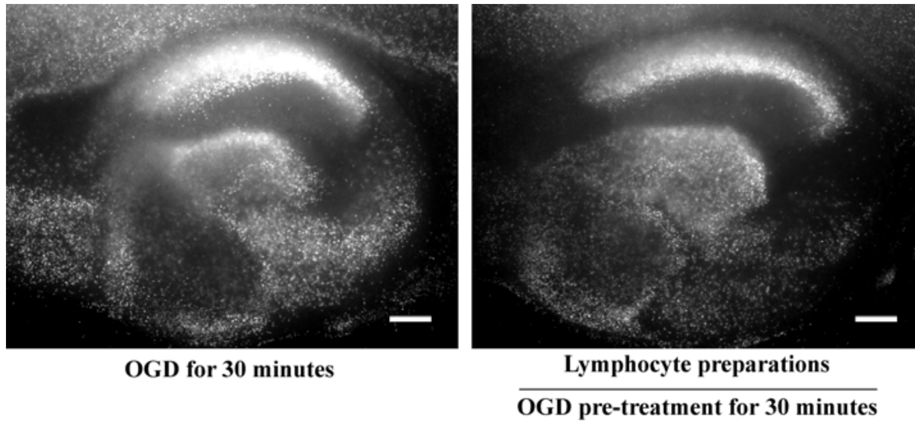
Figure 4.10 Heated conditioned media is not neuroprotective against KA-induced cell death.

Slices were treated as previously described (Figure 4.2). (A) Representative PI fluorescence images of slices treated with KA (300 μM) for 1 hour and incubated in either conditioned media-II or heated conditioned media-II for 18 hoours. (B) Bar chart demonstrating that heated conditioned media-II failed to exhibit neuroprotection against KA-induced neurotoxicity whereas conditioned media-II showed neuroprotection against KA-induced neurotoxicity. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. ***$p < 0.001$ versus KA 300 μM. Scale bar: 200 μm.

4.2.8 Lymphocytes are neuroprotective against OGD

Having observed a neuroprotective role of lymphocytes in KA-induced neurotoxicity, I next wanted to apply this to an *in vitro* model of stroke. I therefore investigated the role of lymphocytes in OGD to compare this with KA-induced neurotoxicity. OSCs were exposed to OGD for 30 minutes before transfer to fresh media containing lymphocyte preparations ($1X10^6$ cells per ml) for 18 hours. Lymphocyte preparations showed neuroprotection against OGD-induced cell death (61.6 ± 3.0 % of 30 mins OGD control; $n = 16$ slices from 4 animals; $p < 0.001$; Figure 4.11) which is similar to the neuroprotection exhibited by lymphocyte preparations in KA-induced neurotoxicity. Next I investigated whether conditioned media-II which was obtained from lymphocytes only is neuroprotective.

(A)



(B)

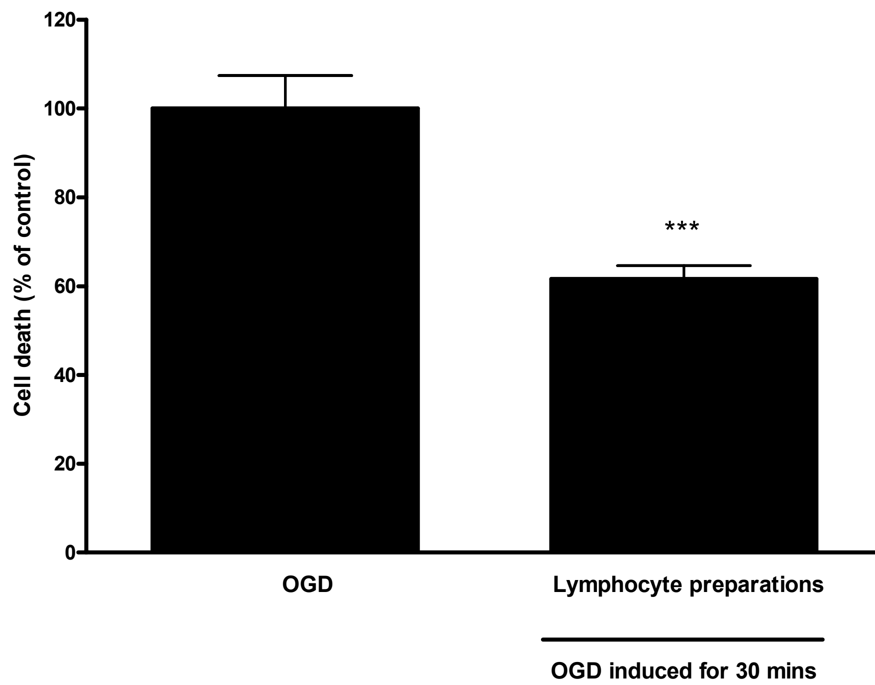


Figure 4.11 Lymphocytes exhibit neuroprotection against OGD-induced cell death.

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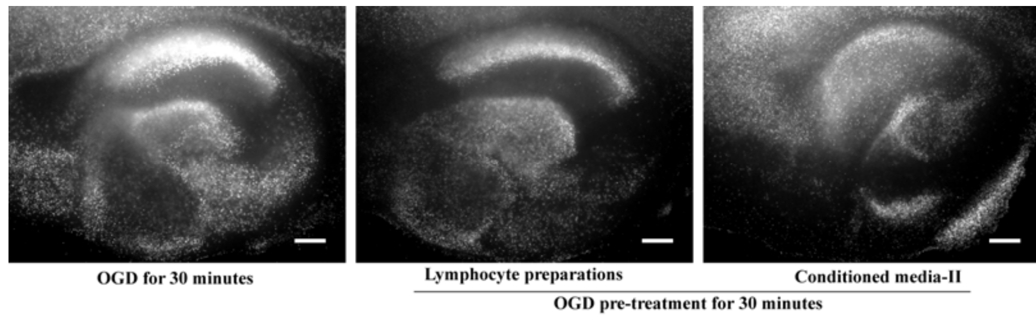
Figure 4.11 Lymphocytes exhibit neuroprotection against OGD-induced cell death. Slices after culturing for 13-15 days in prior to experiments, were subjected to OGD for 30 minutes and co-cultured with 1 X 10^6 lymphocytes for 18 hours in the presence of PI (2 μM). The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images of slices exposed to OGD for 30 minutes and incubated with either fresh culture media or media containing lymphocytes for 18 hours. (B) Bar chart showing that lymphocytes reduced the cell death induced by OGD. All experiments were carried out in at least 4 separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. ***$p < 0.001$ versus 30 mins OGD. Scale bar: 200 μm.

4.2.9 Conditioned media from lymphocyte preparations is neuroprotective against OGD

From the previous results in this chapter, it is clear that lymphocyte-mediated neuroprotection in KA-induced neurotoxicity is contact-independent and the conditioned media is neuroprotective against KA-induced neurotoxicity. To determine whether the observed neuroprotection in OGD by lymphocytes is also contact-independent, I used conditioned media-II (obtained from lymphocyte preparations without exposure to OSCs). OSCs were exposed to OGD for 30 minutes and incubated in conditioned media-II for 18 hours. Conditioned media-II was neuroprotective against OGD (65.9 ± 7.3 % of 30 mins OGD control; $n = 4$ slices from 3 animals; $p < 0.05$; Figure 4.12). No significant difference was found in the level of neuroprotection between lymphocyte preparations and the conditioned media. This shows that the observed neuroprotection is also contact-independent which is similar to KA model.

(A)



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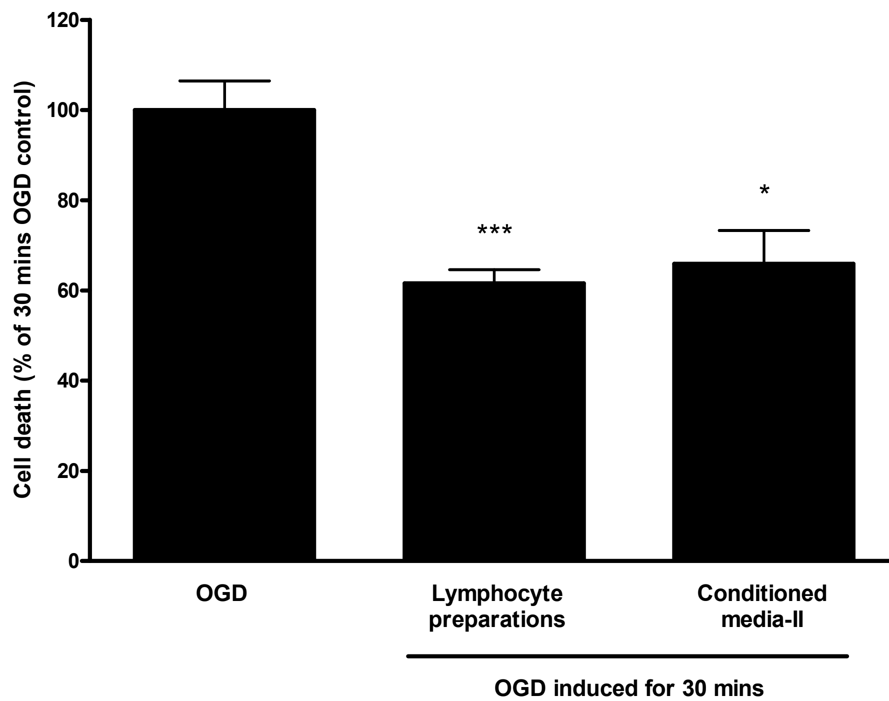


Figure 4.12 Conditioned media from lymphocyte preparations is neuroprotective.

Continued…

Figure 4.12 Conditioned media from lymphocyte preparations is neuroprotective. Slices were exposed to OGD for 30 minutes and incubated in conditioned media-II in the presence of PI (2 μM) for 18 hours. The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images of slices showing cell death in slices exposed to OGD for 30 minutes and incubated with either fresh media or conditioned media-II or media containing lymphocyte preparations for 18 hours. (B) Summary of neuroprotection exhibited by lymphocytes and conditioned media in OGD condition. All experiments were carried out in at least 3 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ versus 30 mins OGD. Scale bar: 200 μm.

4.3 Discussion

In this chapter, I sought to investigate the role of lymphocytes in neurodegenerative diseases. Using two different approaches, I demonstrated that lymphocyte preparations are neuroprotective against both KA-induced toxicity and OGD-induced cell death. Although, various studies have demonstrated the neuroprotective role of T cells (Schwartz, 2005; Beers *et al.*, 2008) and B cells (Fillatreau *et al.*, 2002; Kala *et al.*, 2010) in neurodegenerative diseases, no literature has been found discussing the role of lymphocytes preparations. However, recent *in vivo* study in recombination activating gene 1 (RAG1) knock-out mice (T cells and B cells deficit mice), showed more neurodegeneration with spontaneous recurrent seizures following KA injection in comparison to saline injected RAG1 knock-out mice and wild type (Zattoni *et al.*, 2011). Their study has suggested that adaptive immune response can modulate neuronal damage and spontaneous recurrent seizures in KA-lesioned hippocampus. Therefore, a possible explanation for the neuroprotective response of lymphocyte preparations might be due to the presence of T cells, B cells and other immune cells including macrophages and natural killer cells as a neuroprotective role of these cells have been demonstrated by several studies (Moalem *et al.*, 1999; Hammarberg *et al.*, 2000; Wolf *et al.*, 2002; Schwartz, 2005; Beers *et al.*, 2008). The neuroprotective role of T cells and B cells has been demonstrated in various models of neurodegenerative diseases which are described later in this section. Supporting this argument, flow cytometry revealed that the majority of cells in lymphocyte preparations were found to be T cells.

Next to determine whether T cells were the responsible cells to demonstrate neuroprotection against KA-induced cell death, I purified T cells from lymphocyte preparations. I also demonstrate that purified non-activated and purified activated T cells are neuroprotective. No significant differences were found in the level of neuroprotection between purified non-activated T cells and purified activated T cells which suggested that the observed neuroprotection is independent of the activation state of T cells. Although, the infiltration of non-activated T cells into the CNS has been

shown (Seabrook *et al.*, 1998), no data was found discussing their role in either normal or diseased state of the CNS. The present study has highlighted the potential role for non-activated T cells during neurodegeneration. However, these data must be interpreted with caution because the role of non-activated T cells in *in vitro* and *in vivo* disease models might be different and further investigation is necessary to determine their contribution in the *in vivo* diseased state. The current study has also demonstrated the neuroprotective role of purified activated T cells which corroborate the findings of a great deal of the previous work in this field (Moalem *et al.*, 1999; Wolf *et al.*, 2002; Schwartz, 2005; Beers *et al.*, 2008). When the adoptive transfer of auto-reactive T cells immunised with myelin-basic protein, a self-antigen from EAE-induced mice to the mice with partial optic nerve crush, a model for secondary neurodegeneration, optic nerves were found to survive well and resist the secondary neurodegeneration in comparison to PBS injected mice (Moalem *et al.*, 1999). A similar observation has been demonstrated by Kipnis *et al.* (2002) where the administration of auto-reactive Th-1 cells to EAE-induced or optical nerve crush mice exhibited more neuronal survival in compared to PBS injected controls. The neuroprotective role of T cells has also been demonstrated in T cell and B cell deficit mice, SCID mice (Serpe *et al.*, 1999; Serpe *et al.*, 2003). After facial nerve transaction, SCID mice manifested severe impairment of facial motor neurons which were restored up to wild-type controls after adoptive transfer of wild-type splenocytes containing T and B cells (Serpe *et al.*, 1999; Serpe *et al.*, 2003) suggesting potential role of lymphocytes in protecting neurons from secondary neurodegeneration. In addition, supporting the present data, an *in vitro* study in murine entorhinal-hippocampal brain slices has shown the better survival when incubated with Th-1 and Th-2 cells (Wolf *et al.*, 2002) however, this study didn't induce neurodegeneration. Moreover, differentiation of activated T cells into T_{reg} cell and their contribution in the observed neuroprotection under the present experimental conditions cannot be excluded as it has been illustrated that upon activation by their cognate antigen/MHC-II, $CD4^+$ T cells in the presence of TGF-β and IL-10 or IL-2 are able to differentiate into T_{reg} cells (Nakamura *et al.*, 2001; Chen *et al.*, 2003; Bettelli *et al.*, 2006). Hence, another possible explanation for the observed neuroprotection might be

due to the presence of T_{reg} cells which are suggested to have an immunosuppressive role during neuroinflammation (Liu *et al.*, 2006; Liu *et al.*, 2009; Reynolds *et al.*, 2010). However, the present study has not examined the presence of T_{reg} cells in lymphocyte preparations and a further study could assess their population in lymphocyte preparations.

Further to determine whether non-T cell preparations have any role in the observed neuroprotection, I used non-T cell preparations. After separating T cells from lymphocyte preparations, non-T cells also exhibited neuroprotection against KA-induced neurotoxicity. The analysis of non-T cell preparations by flow cytometer showed that the majority of cells were B cells which suggest that neuroprotection elicited by non-T cells might be due to the presence of B cells. The present findings seem to be consistent with other research which has demonstrated a possible role for B cells in neurodegenerative diseases (Fillatreau *et al.*, 2002; Kala *et al.*, 2010; Berer *et al.*, 2011). The neuroprotective role of B cells has been suggested to be mediated via IL-10 production (Fillatreau *et al.*, 2002; Matsushita *et al.*, 2008; Carter *et al.*, 2011) since, mice lacking IL-10 producing B cells exhibited a disease progression as well as an increase in the number of pathogenic T cells but decrease in the number of T_{reg} cells (Carter *et al.*, 2011). In addition, B cells have been shown to release various growth factors including BDNF, NT-3 and TGF-β and anti-inflammatory cytokines such as IL-4 and IL-10 (Kerschensteiner *et al.*, 1999; Edling *et al.*, 2004; Kala *et al.*, 2010) which can contribute to neuroprotection. Meanwhile, the contribution of other immune cells including macrophages and natural killer cells in neuroprotection cannot be excluded as various studies have shown their neuroprotective role in neurodegenerative diseases (Zhang *et al.*, 1997; Hammarberg *et al.*, 2000; Schwartz and Yoles, 2006; London *et al.*, 2011). A further study with more focus on these cells is therefore suggested.

Having established that lymphocytes are neuroprotective, I investigated whether lymphocytes interact with cells within OSCs to show the observed neuroprotection. This revealed that there is no direct contact between lymphocytes and cells within OSCs. This

suggests that the neuroprotective role of lymphocyte is contact-independent and supports a notion of contact-independent neuroprotection by T cells (Wolf *et al.*, 2002). Wolf *et al.* (2002) found that direct contact of T cells with OSCs was neurotoxic while OSCs co-cultured with T cells separated by insert membranes demonstrated a neuroprotective effect and proposed the notion of contact-independent response of T cells. The current study has further confirmed this notion by using conditioned media to test against KA-induced neurotoxicity. The present data show that conditioned media from lymphocyte preparations is neuroprotective but when the conditioned media was boiled, it failed to exhibit neuroprotection which strongly suggest that soluble mediators released from lymphocytes are proteinaceous. It seems possible that the observed neuroprotection exhibited by soluble mediators might be due to the presence of various neurotrophic factors, growth factors and anti-inflammatory mediators. Several studies have reported that lymphocytes release neurotrophic factors such as BDNF, NT-3 and NT-4/5 (Kerschensteiner *et al.*, 1999; Schuhmann *et al.*, 2005; Heese *et al.*, 2006) and these factors contribute to neuroprotection and repair injured nervous system (Sendtner *et al.*, 1992; Yan *et al.*, 1992; McTigue *et al.*, 1998; Culmsee *et al.*, 2002). In addition to neurotrophic factors, lymphocytes also release neuroprotective cytokines including IL-4 and IL-10 and growth factors like TGF and insulin-like growth factor (IGF) (Zhu *et al.*, 2002; Zhao *et al.*, 2006; Schwartz and Ziv, 2008; Ouyang *et al.*, 2011; Xin *et al.*, 2011). These may underlie the neuroprotection contributed by the conditioned media however, the identification of the exact mediator(s) was not examined in the present study.

The present study has also investigated the role of lymphocytes in an *in vitro* model of stroke (OGD) to compare with the response of lymphocytes in KA-induced neurotoxicity as infiltration of lymphocytes into the CNS has been reported by several studies in stroke (Schroeter *et al.*, 1994; Jander *et al.*, 1995; Campanella *et al.*, 2002; Stevens *et al.*, 2002) and *in vivo* multiphoton real-time live imaging has further supported the infiltration of lymphocytes predominantly T cells into the brain during stroke (Ortolano *et al.*, 2010; Fumagalli *et al.*, 2011). Although, various *in vivo* studies (Hurn *et al.*, 2007; Liesz *et al.*, 2011) have reported the neurotoxic effect of lymphocytes

in stroke, the present data has shown that lymphocytes are neuroprotective in OGD under the present experimental conditions. To the best of my knowledge, this is the first time that the neuroprotective role of lymphocytes in an *in vitro* model of stroke has been shown. Another important finding of the current study is that the lymphocyte-mediated neuroprotection is contact-independent since conditioned media also exhibited neuroprotection against OGD-induced cell death. Supporting these findings, several *in vivo* studies have demonstrated the neuroprotective role of lymphocytes in stroke (Frenkel *et al.*, 2005; Planas and Chamorro, 2009; Ren *et al.*, 2011). The infarct size of the brain is found to be significantly reduced in the mice with adoptive transfer of MOG-specific $CD4^+$ T cells (Frenkel *et al.*, 2005). A possible explanation for the neuroprotective role of lymphocytes under the present study might be due to the presence of IL-10 and TGF-β released by T cells and B cells. IL-10 has also been suggested to be a modulator of activated macrophage/microglia and astrocytes thus limiting secondary inflammatory processes (Arumugam *et al.*, 2005) and also reported to be protective against glutamate toxicity (Bachis *et al.*, 2001). IL-10 secreted by $CD4^+$ T cells has been suggested to be responsible for reduction in the infarct size of the brain during stroke (Frenkel *et al.*, 2003; Frenkel *et al.*, 2005). In addition, B cells have also been found to be the major IL-10 producing cells which help in reducing CNS inflammation and neurological deficit during stroke (Ren *et al.*, 2011) since the adoptive transfer of B cells from wild-type to B cell deficient mice prevented from ischemia while transfer of B cells from $IL\text{-}10^{-/-}$ mice to B cells deficient mice fails to prevent it. The low level of IL-10 in elderly patient with the history of stroke in comparisons to elderly people without stroke further support the potential role of IL-10 during stroke (van Exel *et al.*, 2002; Vila *et al.*, 2003) as lower plasma concentrations of IL-10 were correlated with the patient having the worst neurological functions following stroke (Vila *et al.*, 2003). Recently, the role of T_{reg} cells has been highlighted in stroke. T_{reg} cells have been suggested to be neuroprotective in the stroke (Liesz *et al.*, 2009; Planas and Chamorro, 2009) and reported to be involved in reducing the production of pro-inflammatory cytokines including TNF-α, INF-γ and IL-1β, inhibition of neutrophils infiltration into the brain and modulate the activation of microglia in the ischemic brain

(Liesz *et al.*, 2009). Leisz *et al.* (2009) further suggested that the neuroprotective response of T_{reg} cells is mediated via IL-10 since adoptive transfer of IL-$10^{-/-}$ T_{reg} cells fails to reduce the infarct volumes in comparison to their control mice. Therefore, it is very likely that IL-10 could be one of the factors that contribute to the neuroprotection observed under the present experimental conditions.

Another possible explanation might be due to the presence of TGF-β as several studies have shown the neuroprotective effect of TGF-β (Prehn *et al.*, 1993; Henrich-Noack *et al.*, 1996; Buisson *et al.*, 2003). There is evidence for the expression of TGF-β1 in the brain after ischemia (Krupinski *et al.*, 1996; Ruocco *et al.*, 1999; Buisson *et al.*, 2003) and it can act as a neuroprotective cytokine to limit the extent of injury (Ruocco *et al.*, 1999; Arumugam *et al.*, 2005). The administration of TGF-β1 in animal models of cerebral ischemia has demonstrated the significant reduction in the infarct volume (Prehn *et al.*, 1993; McNeill *et al.*, 1994; Henrich-Noack *et al.*, 1996) which further supports the neuroprotective role of TGF-β. In the ischemic brain, TGF-β1 protects against the injury of the brain mediated by excitotoxic and ischemic injury (Prehn *et al.*, 1993; McNeill *et al.*, 1994; Henrich-Noack *et al.*, 1996; Arumugam *et al.*, 2005). Further, it has also been suggested that TGF-β1 can reduce cytotoxicity mediated by microglia and deactivate macrophages (Tsunawaki *et al.*, 1988; Merrill and Zimmerman, 1991; Flanders *et al.*, 1998) thus inhibiting inflammatory responses.

There are, however, other possible explanations for the observed neuroprotection. Anti-inflammatory cytokines including IL-4, IL-5 and IL-13 produced by Th-2 cells (Mosmann *et al.*, 1986; Arumugam *et al.*, 2005; Kaiko *et al.*, 2008; Wilson *et al.*, 2009) are also potential mediators which can contribute to neuroprotection. Moreover, several other studies demonstrated that different neurotrophic factors (such as BDNF, NT3 and NT4/5) and growth factors (such as TGF and IGF) being released by lymphocytes (Kerschensteiner *et al.*, 1999; Schuhmann *et al.*, 2005; Heese *et al.*, 2006) which have neuroprotective properties (McTigue *et al.*, 1998; Culmsee *et al.*, 2002; Ouyang *et al.*,

2011). Taken together, these findings suggest that these factors might have potential role in contributing to neuroprotection under the present experimental conditions.

In summary, the present data has suggested that lymphocyte preparations are neuroprotective regardless of the activation state of T cells. The current data demonstrates the neuroprotective role of non-activated T cells and activated T cells against KA-induced neurotoxicity. Interestingly, the present study has shown that non-T cells are also neuroprotective and there is no significant difference in the level of neuroprotection when compared with purified T cells suggesting that the neuroprotection exhibited by T cells is independent of their activation state. Moreover, the present data showed evidence for contact-independent neuroprotection exhibited by lymphocytes and soluble mediators released by lymphocytes are responsible for the observed neuroprotection. Taken together, these findings suggest that lymphocytes are neuroprotective and can effectively reduce cell death in an *in vitro* model of neurodegeneration. In addition, further investigation in an *in vitro* model of stroke to determine the role of lymphocytes revealed a neuroprotective response against OGD-induced cell death. The present study has also shown that lymphocyte-mediated neuroprotection is contact-independent in the OGD model as similar to the KA model. However, as OSCs have a heterogeneous cells population, the involvement of other non-neuronal cells including astrocytes and microglia in lymphocyte-mediated neuroprotection cannot be excluded in both neurodegenerative diseases models.

5 Determining the underlying mechanism(s) of lymphocyte-mediated neuroprotection

In the previous chapter, I showed that lymphocytes are neuroprotective against KA-induced neurotoxicity and OGD-induced cell death. Interestingly, I also showed that in both models for neurodegenerative diseases, the lymphocyte-mediated neuroprotection is contact-independent and soluble mediators released from lymphocytes are responsible for the observed neuroprotection. As OSCs contain a heterogeneous cell population including neurons, astrocytes and microglia, it is likely that these cells might have a role in the observed neuroprotection. There are other cells including pericytes, endothelial cells and ependymal cells present in the nervous system apart from neurons and glial cells but normally these cells don't survive in the present culture conditions (Weis *et al.*, 2001; Moser *et al.*, 2003). So, it is possible that these cells don't have significant effect on the culture system. Various studies have demonstrated the neuroprotective role of lymphocytes (Moalem *et al.*, 2000; Wolf *et al.*, 2002; Garg *et al.*, 2008) but there is little data about the underlying cellular signalling mechanism(s) of lymphocyte-mediated neuroprotection. Therefore, I here investigated which cell types are involved in the lymphocyte-mediated neuroprotection and the underlying cellular signalling mechanism(s), using two different models of neurodegenerative diseases: the KA model and the OGD model.

5.1 Introduction

As previously described, in addition to neurons organotypic slice cultures contain astrocytes and microglia (Coltman and Ide, 1996; Czapiga and Colton, 1999; Huuskonen *et al.*, 2005) which might have important role in the lymphocyte-mediated neuroprotection observed in Chapter 4. Therefore, taking this fact into consideration, I have investigated the possible role of astrocytes in the observed neuroprotection. As described earlier in the introduction chapter, astrocytes, star-shaped cells, predominant in the CNS glial cell population and form a tight junction of the blood-brain barrier

(BBB) along with the endothelial cell lining of the blood vessels (Wolburg *et al.*, 1994; Abbott *et al.*, 2006; Persidsky *et al.*, 2006; Bernacki *et al.*, 2008). These cells provide a micro-architecture of grey matter having separate domains, referred to as, 'astrocytic domains' (Bushong *et al.*, 2002; Bushong *et al.*, 2004; Allaman *et al.*, 2011). Under normal physiological conditions, these cells regulate neuronal function by integrating neuronal firing and synaptic networks via cytoplasmic calcium (Ca^{2+}) waves (Dani *et al.*, 1992; Pasti *et al.*, 1997; Santello and Volterra, 2009) and also maintain ionic composition, pH and remove excess neurotransmitters from the synaptic cleft (Andersen *et al.*, 2000; Matsuura *et al.*, 2002; Sofroniew and Vinters, 2010).

It is also evident that astrocytes play a crucial role in neurodegenerative diseases including AD, MS, PD and stroke (Seifert *et al.*, 2006; Nair *et al.*, 2008; Rodriguez *et al.*, 2008; Allaman *et al.*, 2010; Chao *et al.*, 2010; Gu *et al.*, 2010) and have been suggested to be immnocompetent cells that can modulate neuroinflammation (Brambilla *et al.*, 2005; van Loo *et al.*, 2006; Belanger and Magistretti, 2009). Under pathological conditions, astrocytes become activated (Sofroniew and Vinters, 2010) and release various cytokines, chemokines, reactive oxygen species and growth factors which can be either neuroprotective or neurotoxic (Sofroniew, 2005; Farina *et al.*, 2007) depending on their conditions. Activated astrocytes release deleterious reactive oxygen species and form glial scar which can obstruct regeneration of axon and neurite outgrowth (Silver and Miller, 2004; Sofroniew, 2005). On the other hand, scar formations restrict inflammation by surrounding the inflammation site and separate it from immediate viable neurons such that those neurons get protected from the inflammatory responses (Faulkner *et al.*, 2004; Sofroniew, 2005; Sofroniew and Vinters, 2010). Due to this fact, the role of activated astrocytes is still elusive. However, there is evidence for the neuroprotective role of astrocytes (Desagher *et al.*, 1996; Faulkner *et al.*, 2004; Dhandapani *et al.*, 2005; Greenwood and Bushell, 2010). An *in vitro* study on neuronal cultures has demonstrated that neurons when co-cultured with astrocytes showed neuroprotection against peroxide-induced toxicity (Desagher *et al.*, 1996). Similar results have also been demonstrated in organotypic hippocampal slice cultures with

conditioned media from astrocytes culture where NMDA-induced neurotoxicity was significantly reduced and also maintained hippocampal cytoarchitecture (Hailer *et al.*, 2001). Astrocytes have also been shown to modulate PAR-2 mediated neuroprotection against KA-induced cell death (Greenwood and Bushell, 2010). In addition, it has been shown that astrocytes can inhibit microglial activation by suppressing reactive oxygen species levels thus protecting neurons from inflammatory responses (Min *et al.*, 2006). From these findings, it is evident that astrocytes can be one of the key players in modulating outcomes of pathology during neurodegenerative diseases. Hence, the role of astrocytes in the observed neuroprotection (see Chapter 4) cannot be excluded.

Although a considerable amount of literature has been published on the neuroprotective role of lymphocytes (Moalem *et al.*, 2000; Schwartz, 2005; Beers *et al.*, 2008), little attention has been paid to the underlying cellular signalling mechanism(s) of the observed neuroprotection. This shows an explicit need to explore how the observed neuroprotection has been mediated by lymphocytes. Therefore, I investigated MAP kinase signalling pathways, as these pathways are involved in various cellular functions including cell differentiation, cell movement, cell division and cell death (Schaeffer and Weber, 1999; Chang and Karin, 2001; Dong *et al.*, 2002). MAP kinase signalling pathways are one of the most extensively studied signal transduction pathways. This signalling pathway responds to physical and chemical stresses thereby controlling cell survival and adaptation by regulating cytoplasmic activities and gene expression (Whitmarsh *et al.*, 1998; Schaeffer and Weber, 1999; Chang and Karin, 2001; Turjanski *et al.*, 2007). The activity of MAP kinases is regulated via three kinase cascades comprising of MAP kinase, MAP kinase kinase (MAPKK or MEK), and MAPKK kinase (MAPKKK or MEKK) (English *et al.*, 1999; Dhanasekaran *et al.*, 2007). There are three families of MAP kinases; extracellular-signal regulated protein kinases (ERK), p38 MAP kinases and c-Jun NH_2-terminal kinases (JNK). Among these three groups of MAP kinases, generally, ERK is involved in cell differentiation and growth while JNK and p38 kinase respond to stress including inflammation and apoptosis (Xia *et al.*, 1995; English *et al.*, 1999; Schaeffer and Weber, 1999; Raman *et al.*, 2007). The ERK family

consists of ERK1, ERK2 and ERK5 isoforms. ERK1 and ERK2 are activated by upstream kinases MEK1 and MEK2 whereas ERK5 is activated via MEK5 (Seger and Krebs, 1995; Cobb, 1999; Chang and Karin, 2001; Harper and Wilkie, 2003). The p38 MAP kinase family have four isoforms including p38α, p38β, p38γ and p38δ and among the four p38 isoforms, p38α and p38β are expressed ubiquitously while p38γ is expressed in skeletal muscle and p38δ is expressed in lung, kidney, testis, pancreas and small intestine (Jiang *et al.*, 1996; Lechner *et al.*, 1996; Li *et al.*, 1996; Kumar *et al.*, 1997; Ono and Han 2000). The upstream kinases that normally activate p38 MAP kinases are MKK3 and MKK6 (Ono and Han, 2000; Harper and Wilkie, 2003) but p38 MAP kinases have also been reported to be activated by MKK4 (Jiang *et al.*, 1997). The third MAP kinase family, JNK family which is also known as stress-activated protein kinases (SAPK1 family), include 10 isoforms encoded by three genes, JNK1 (4 isoforms), JNK2 (4 isoforms) and JNK3 (2 isoforms) (Gupta *et al.*, 1996; Davis, 2000; Johnson and Nakamura, 2007). JNK1 and JNK2 are widely expressed whereas JNK3 is primarily expressed in the brain (Mohit *et al.*, 1995; Harper and Wilkie, 2003). The JNK family are activated by their upstream kinases, MKK4 and MKK7 (Davis, 2000; Johnson and Nakamura, 2007).

Normally, in the CNS, ERKs are responsible for cell growth, cell differentiation and regulation of neuronal cell fate and plasticity (Cruz and Cruz, 2007). ERK activation has been correlated with the survival of cortical neurons under hypoxic conditions (Hetman *et al.*, 1999; Sun *et al.*, 2008). Furthermore, it has also been demonstrated that activation of ERK was responsible for neuroprotection in neuronal culture against cytosine arabinoside (araC)-induced toxicity (Xue *et al.*, 2000) and ERKs contribute to neuronal survival by inhibiting JNK/p38 MAP kinases (Xia *et al.*, 1995). However, ERKs have been reported to be involved in activation of apoptotic/necrotic pathways in certain neurodegenerative diseases including AD, PD and stroke (Pettmann and Henderson, 1998; Namura *et al.*, 2001; Cheung and Slack, 2004). An *in vitro* study in a culture model has shown that the persistent activation of ERK contributed to glutamate-induced oxidative neurotoxicity which was prevented by MEK inhibitors (Stanciu *et al.*, 2000).

Similarly, in an animal model of stroke, upregulation of ERK activity after ischaemia and reperfusion has been found to be toxic leading to cell death (Alessandrini *et al.*, 1999; Namura *et al.*, 2001). On the other hand, p38 MAP kinases and JNKs are considered to be potent players in cell death (Mielke and Herdegen, 2000). Various stimuli including cytokines, stress environment, growth factors and pathogens can activate p38 MAP kinases (Ono and Han, 2000). The activation of p38 MAP kinases has been demonstrated in neuronal cultures leading to cell death after withdrawal of growth factors which was reversed by a p38 MAP kinase inhibitor (Heidenreich and Kummer, 1996; Horstmann *et al.*, 1998). This suggests that the activation of p38 MAP kinase can be neurotoxic. In addition, p38 MAP kinase has been suggested to be a key player in various neurodegenerative diseases (Walton *et al.*, 1998; Irving *et al.*, 2000; Du *et al.*, 2001). The accumulation of tau proteins and release of pro-inflammatory mediators in an animal model of AD has been correlated with the activation of p38 MAP kinase (Savage *et al.*, 2002; Culbert *et al.*, 2006). As mentioned earlier, JNKs play an important role in apoptosis and inflammation (Chen *et al.*, 1996; Benakis *et al.*, 2010) and can also be activated by various extracellular stimuli including ultraviolet light, heat shock, withdrawal of growth factors and a number of toxins (Xia *et al.*, 1995; Bruckner *et al.*, 2001; Harper and Wilkie, 2003; Johnson and Nakamura, 2007). The activation of these kinases has been implicated in various neurodegenerative diseases (Takagi *et al.*, 2000; Morishima *et al.*, 2001; Savage *et al.*, 2002; Klegeris *et al.*, 2008). Savage *et al.* (2002) have demonstrated that the JNK/p38 MAP kinase-mediated accumulation of tau proteins induce neurotoxicity in an animal model of AD. It has also been shown that JNK inhibiton in cerebral ischemia reduces infarct volume (Benakis *et al.*, 2010) which suggests a critical role of JNK in ischaemia. Moreover, a recent study has shown the up-regulation of all three MAP kinases in the brain of KA treated mice (De Lemos *et al.*, 2010). When taken together, these findings suggest that the MAP kinase signalling pathways may be involved in the lymphocyte-mediated neuroprotection. I therefore investigated MAP kinase signalling pathway to determine whether these pathways underlie the observed neuroprotection.

In addition to MAP kinases, phosphotidylinositol-3 (PI-3) kinases might also have an important role in the observed neuroprotection as neurite growth and neuritogenesis mediated by growth factors including BDNF and neurotrophins are involved in the activation of the MAP kinase pathways and the PI-3 kinase pathways (Patapoutian and Reichardt, 2001; Tang, 2003). These kinases also contribute in the regulation of cellular functions including cellular differentiation, growth, proliferation, survival, intracellular trafficking and glucose haemostasis (Fry, 1994; Rameh and Cantley, 1999; Foster *et al.*, 2003). The PI-3 kinase family include Class-I PI-3 kinases, Class-II PI-3 kinases and Class-III PI-3 kinases of which Class-I PI-3 kinases have been most extensively studied (Brown and Shepherd, 2001). Several studies have demonstrated the activation of PI-3 kinase by various growth factors including nerve growth factor and TGF-β lead to neuronal survival and protect from apoptosis (Culmsee *et al.*, 2002; Dhandapani *et al.*, 2005). These kinases produce inositol lipids upon the activation of growth factor receptors and act as a secondary messenger thereby mediating various cellular activities (Stein and Waterfield, 2000; Katso *et al.*, 2001; Foster *et al.*, 2003). These PI-3 kinase lipid products are not in detectable levels in unstimulated cells but get upregulated in response to cell stimulation by growth factors (Irvine, 1992; Fry, 1994; Katso *et al.*, 2001; Cantley, 2002). The activation of PI-3 kinases have been suggested to be important for growth factor mediated neurite growth since PI-3 kinase inhibiton in neuronal cell line culture inhibited the growth (Kimura *et al.*, 1994). PI-3 kinases have also been implicated in inflammatory responses (Wymann *et al.*, 2003) along with various neurodegenerative diseases including AD and PD (Zubenko *et al.*, 1999; Yoshimura *et al.*, 2006; Timmons *et al.*, 2009) and are a target for a drug development (Stein and Waterfield, 2000). With these findings, it can be postulated that PI-3 kinases are likely to be involved in the lymphocyte-mediated neuroprotection since lymphocytes release various growth factors, cytokines and chemokines (Abbas *et al.*, 1996; Moalem *et al.*, 2000; Wan and Flavell, 2009) that may affect PI-3 kinase signalling. Hence, I also investigated the PI-3 kinase signalling pathway in the present study. I used western blotting and pharmacological treatment to address MAP kinase signalling pathways

whereas a pharmacological approach was taken to investigate the role of PI-3 kinase signalling pathways.

5.2 Results

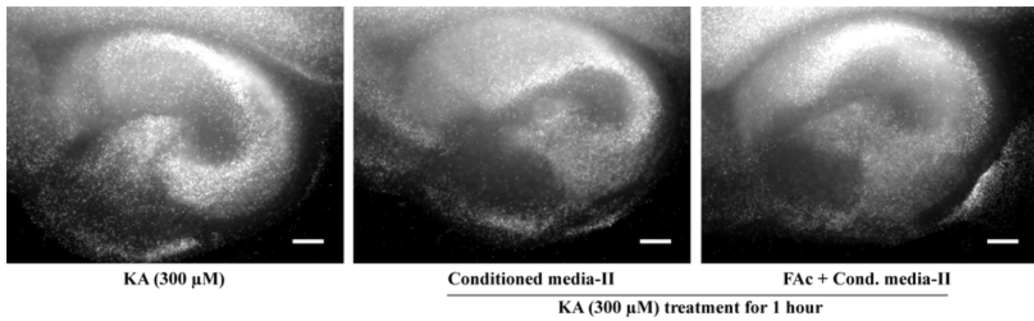
5.2.1 Inhibition of astrocytic function blocks the observed neuroprotection in KA-induced cell death

To investigate the role of astrocytes in the lymphocyte-mediated neuroprotection, astrocytic function was selectively inhibited using FAc, the glial cell metabolic inhibitor (Swanson and Graham, 1994; Fonnum *et al.*, 1997). It has been proposed that FAc-mediated inhibition of astrocytic function is due to the inhibition of Kreb's cycle enzyme aconitase by FAc (Fonnum *et al.*, 1997). Our laboratory group has previously shown that pre-treatment of slices for 3 hours with FAc has no effect on the level of cell death induced by KA (Greenwood and Bushell, 2010). My preliminary data has also shown that pre-treatment of slices with FAc alone was not toxic (mean fluorescent value = 3.16 and 2.76). However, the inhibition of astrocytic function was required throughout the experimental period in the present study to determine their role in the neuroprotection mediated by conditioned media. In addition, it was also important to assess whether FAc can modulate KA-induced toxicity after 18 hours incubation in the presence of FAc. The total duration of FAc pre-treatment was 3 hours, so slices were pre-treated with FAc (10 μM) for 2 hours and were subsequently treated with KA (300 μM) for 1 hour in the presence of with FAc and incubated in fresh culture media containing PI (2 μM) for 18 hours in the presence of FAc. It was then compared with those slices treated with KA alone for 1 hour and incubated in fresh culture media for 18 hours with PI. Quantification of PI uptake showed that there were no significant differences in the level of cell death between FAc treated slice and untreated slice (111.7 ± 4.4 % of the KA 300 μM control; n = 11 slices from 9 animals; Figure 5.1), demonstrating that FAc does not induce toxicity to OSCs even after 18 hours of incubation.

Next, I investigated the role of astrocytes in the neuroprotection mediated by soluble factors from lymphocytes to determine whether the presence of the glial cell metabolic inhibitor has any effect on the observed neuroprotection. After pre-treatment with FAc

for 2 hours, OSCs were further treated with KA for 1 hour in the presence of FAc and incubated in conditioned media-II with PI in the presence of FAc for 18 hours. Quantification of PI fluorescence images of slices treated with FAc revealed that cell death was significantly higher (100.3 ± 3.2 % of the KA 300 µM control; n = 21 slices from 10 animals; Figure 5.1) when compared with OSCs which were incubated in the conditioned media alone (74.4 ± 4.8 % of the KA 300 µM control; n = 27 slices from 12 animals; $p < 0.01$). This finding suggests that the inhibition of astrocytic function may reduce the neuroprotection mediated by the soluble factors released by lymphocytes.

(A)



(B)

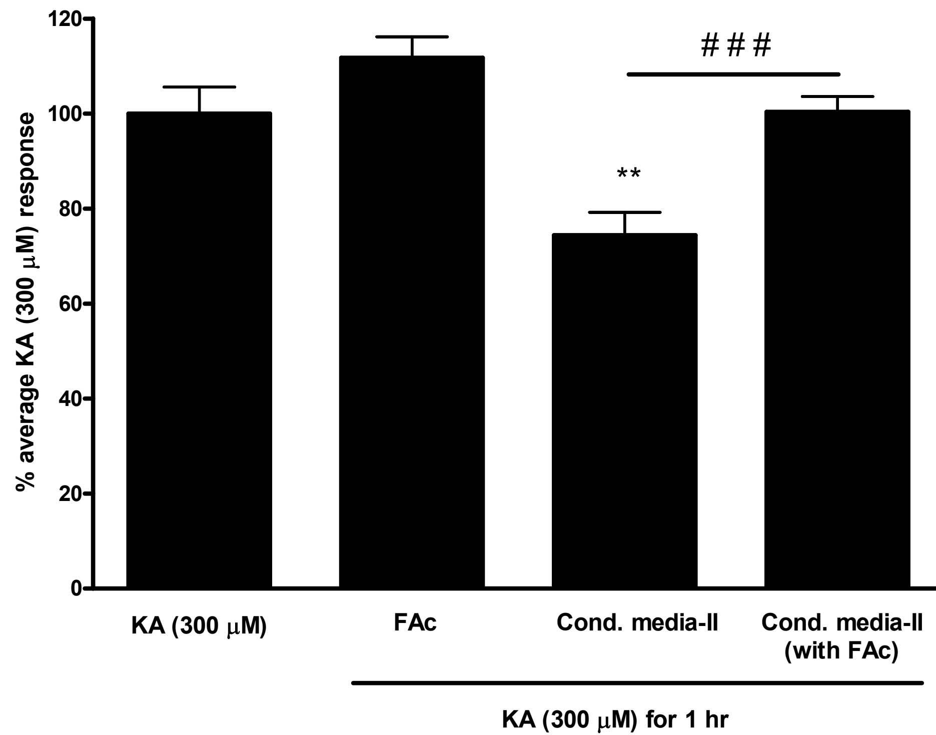


Figure 5.1 The neuroprotection mediated by conditioned media from lymphocytes is blocked following the inhibition of astrocytic function in the KA model.

Continued…

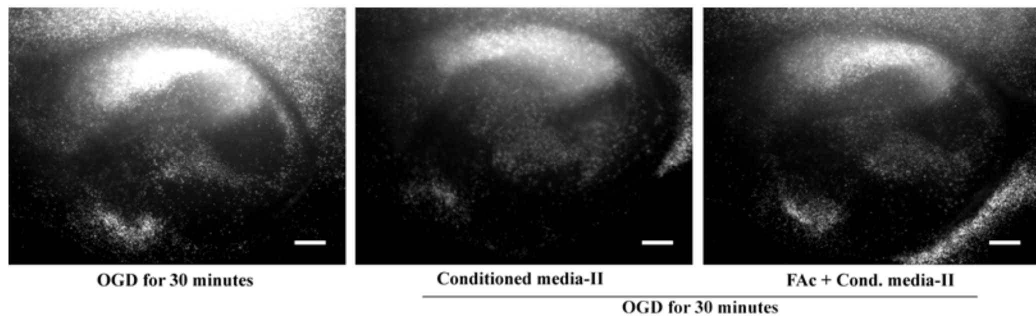
Figure 5.1 The neuroprotection mediated by conditioned media from lymphocytes is blocked following the inhibition of astrocytic function in the KA model.

Slices from 4-7 days old mouse pups were cultured for 13-15 days prior to experiments. Slices were pre-treated with FAc for 2 hours and subsequently treated for 1 hour with KA in the presence of FAc which were then incubated in conditioned media-II for 18 hours either in the presence or absence of FAc with PI. The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices treated with KA following either FAc pre-treatment or without treatment which were then incubated in conditioned media-II either in the presence or absence of FAc for 18 hours. Control slices were treated with KA (300 µM, 1 hr) in the absence of FAc and incubated in fresh culture media for 18 hours (B) Bar chart illustrating the significant reversal of neuroprotection after inhibition of astrocytic function. All experiments were carried out in at least 9 separate animal preparations. Data presented as mean ± SEM. $^{**}p < 0.01$ versus 300 µM KA; # # #$p < 0.001$ versus Cond media-II with FAc. Scale bar: 200 µm.

5.2.2 The neuroprotection mediated by the conditioned media from lymphocytes is independent of astrocytic function in OGD-induced cell death

Having established that astrocytes play a key role in lymphocyte-mediated neuroprotection in KA-induced cell death, I also investigated the role of astrocytes under OGD condition. As described earlier, total duration of FAc pre-treatment was 3 hours, following pre-treatment with FAc for 2 hours 30 minutes, OSCs were subjected to OGD for 30 minutes in the presence of FAc which were then incubated in conditioned media-II containing PI with FAc for 18 hours. Contrary to the result observed in KA-induced cell death, FAc failed to block the lymphocyte-mediated neuroprotection in OGD-induced cell death (56.8 ± 5.9 % of the 30 mins OGD control; n = 5 slices from 4 animals; $p < 0.05$; Figure 5.2) when compared to slices incubated in the conditioned media alone without FAc treatment (65.9 ± 6.0 % of the 30 mins OGD control; n = 4 slices from 3 animals; $p < 0.05$). There was no significant difference in the level of neuroprotection between FAc treated slices and untreated slices which suggest that the neuroprotection mediated by soluble mediators is independent of astrocytic function in OGD-induced cell death.

(A)



(B)

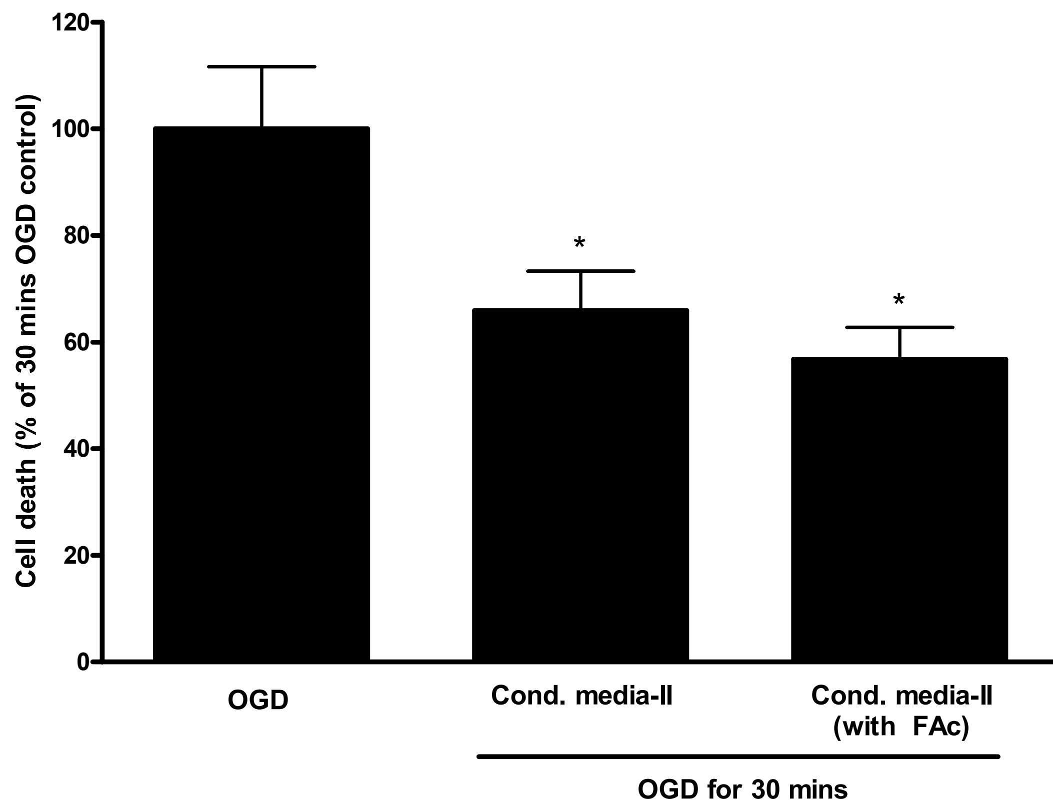


Figure 5.2 Neuroprotection is independent of astrocytic function in the OGD model.

Continued…

Figure 5.2 Neuroprotection is independent of astrocytic function in the OGD model.

Slices were cultured for 13-15 days prior to experiments. Slices after FAc treatment for 2 hours 30 minutes were subjected to OGD for 30 minutes in the presence of FAc (10 μM) for 30 minutes and incubated in conditioned media-II with PI for 18 hours in the presence of FAc. The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in FAc pre-treated slices which were incubated in conditioned media-II either in the presence or absence of FAc for 18 hours. Control slices were treated in OGD condition for 30 minutes and incubated in fresh culture media for 18 hours. (B) Bar chart showing no alteration in the observed neuroprotection in OGD-induced cell death when compared with conditioned media-II alone. All experiments were carried out in at least 3 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$ versus 300 μM KA. Scale bar: 200 μm.

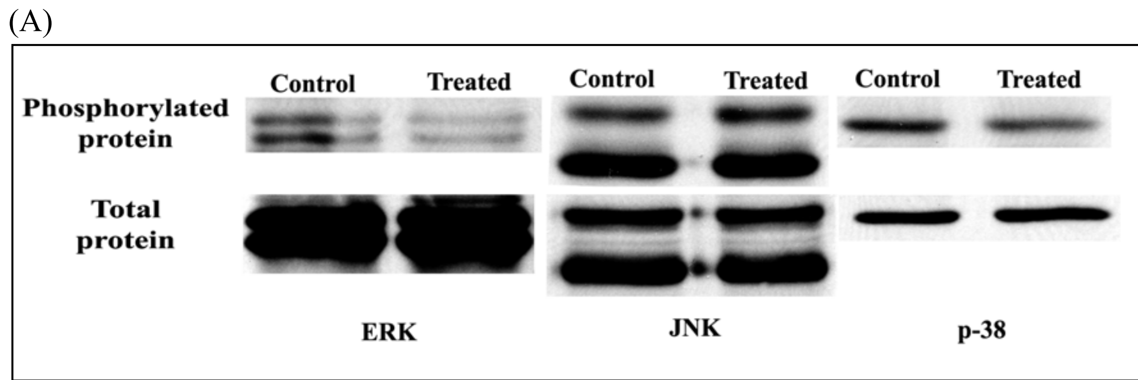
5.2.3 Inhibition of ERK and p38 MAP kinase pathways underlies the lymphocyte-mediated neuroprotection

After establishing the neuroprotective role of lymphocytes under the present experimental conditions and demonstrating a potential role for astrocytes in the neuroprotection against KA-induced cell death, I next sought to investigate the underlying cellular signalling associated with protection using western blotting. Immunoblotting of slices was carried out after incubating OSCs with lymphocyte preparations for 18 hours and compared with slices incubated in fresh culture media alone for 18 hours. The present data showed reduction in ERK-1/2 and p38 MAP kinase activity without affecting JNK-1/2 activity (Figure 5.3). ERK-1/2 activity was reduced to 66.2 ± 7.8 % of control ($n = 5$; $p < 0.05$ compared to control) and the p38 MAP kinase activity was inhibited to 64.0 ± 4.5 % of control ($n = 4$; $p < 0.05$ compared to control). However, the JNK-1/2 activity was not altered (108.5 ± 12.6 % of control; $n = 5$; $p > 0.05$ compared to control).

Next I used a pharmacological approach to determine whether the inhibition of ERK and p38 MAP kinase underlie the lymphocyte-mediated neuroprotection. Here, MAP kinase inhibitors were used to mimic lymphocytes-induced reduction in MAP kinase activity. OSCs treated with KA (300 µM, 1 hr) were incubated in fresh media with PI (2 µM) in the presence of MAP kinase inhibitors, U0126 (20 µM; an inhibitor of ERK) and SB203580 (100 µM; an inhibitor of p38 MAP kinase) for 18 hours. Alternatively, slices were exposed to OGD for 30 minutes prior to transfer into fresh media containing MAP kinase inhibitors, U0126 and SB203580 with PI and incubated for 18 hours. In agreement with western blotting data, quantification of PI images showed that in the KA model, MAP kinase inhibitors significantly reduced KA-induced cell death (79.4 ± 1.7 % of the KA 300 µM control plus vehicle; $n = 11$ slices from 6 animals; $p < 0.001$; Figure 5.4). Similarly, MAP kinase inhibitors also significantly inhibited OGD-induced cell death (66.3 ± 5.8 % of the 30 mins OGD control plus vehicle; $n = 9$ slices from 4

animals; $p < 0.01$; Figure 5.5). Thus, these findings suggested that the inhibition of ERK and p38 MAP kinase underlie the lymphocyte-mediated neuroprotection.

(A)



(B)

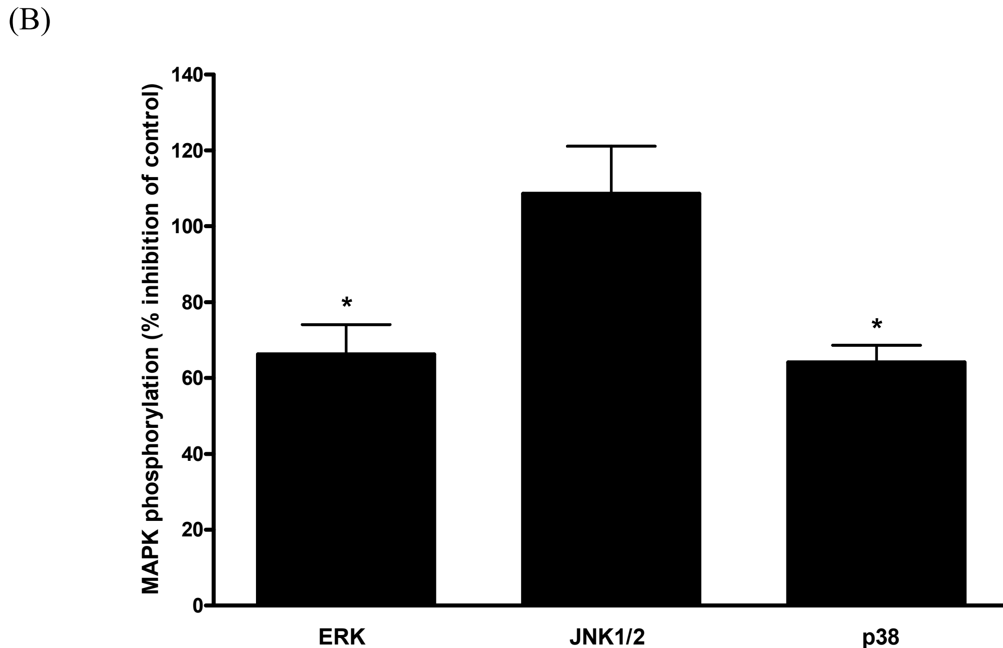
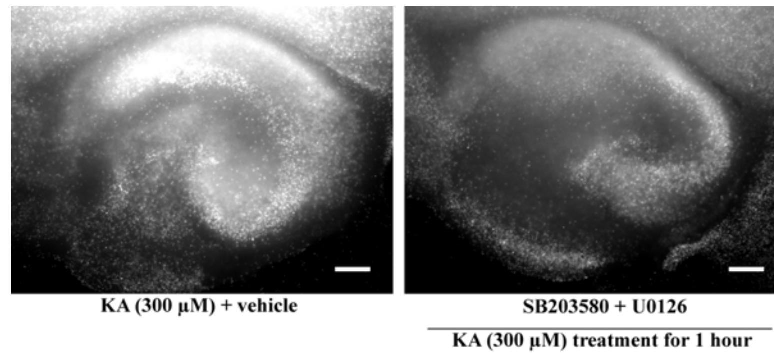


Figure 5.3 Inhibition of MAP kinase pathways was observed in the lymphocyte-mediated neuroprotection.

Slices obtained from C57Bl6 mice were cultured for 13-15 days prior to experiments. Slices were incubated with lymphocyte preparations for 18 hours and western blotting of slices was carried out to investigate Map kinase signalling pathways. (A) Representative blots showing phosphorylated and total MAP kinases. Control slices were incubated in fresh culture media and treated slices were co-cultured with lymphocyte preparations for 18 hours. (B) Quantification of blot showing a decrease in ERK and p38 MAP kinase activities. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$ versus control slice.

(A)



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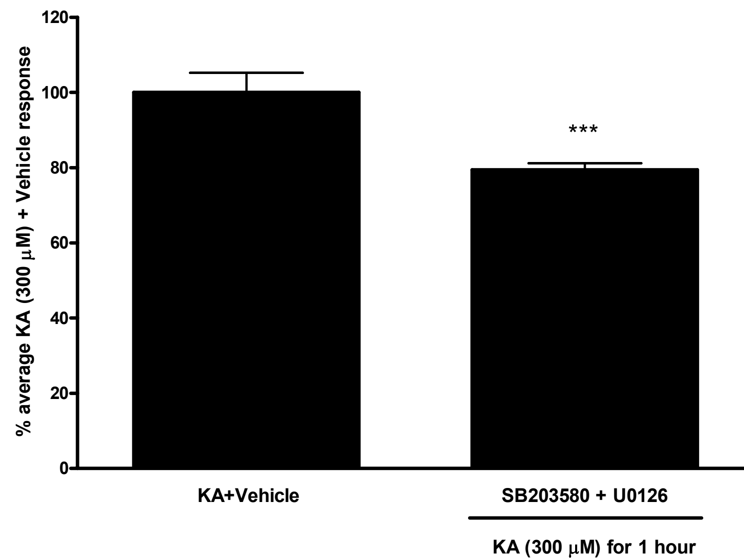


Figure 5.4 Pharmacological inhibition of MAP kinases inhibit KA-induced cell death.

Slices were treated with KA (300 µM, 1 hr) and incubated in fresh culture media containing MAP kinase inhibitors with PI (2 µM) for 18 hours. The PI images were visualised using fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices treated with KA and incubated in fresh culture media with either vehicle or MAP kinase inhibitors for 18 hours. (B) Bar chart showing a reduction in cell death by MAP kinase inhibitors. All experiments were carried out in at least five separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. ***$p < 0.001$ versus 300 µM KA + vehicle. Scale bar: 200 µm.

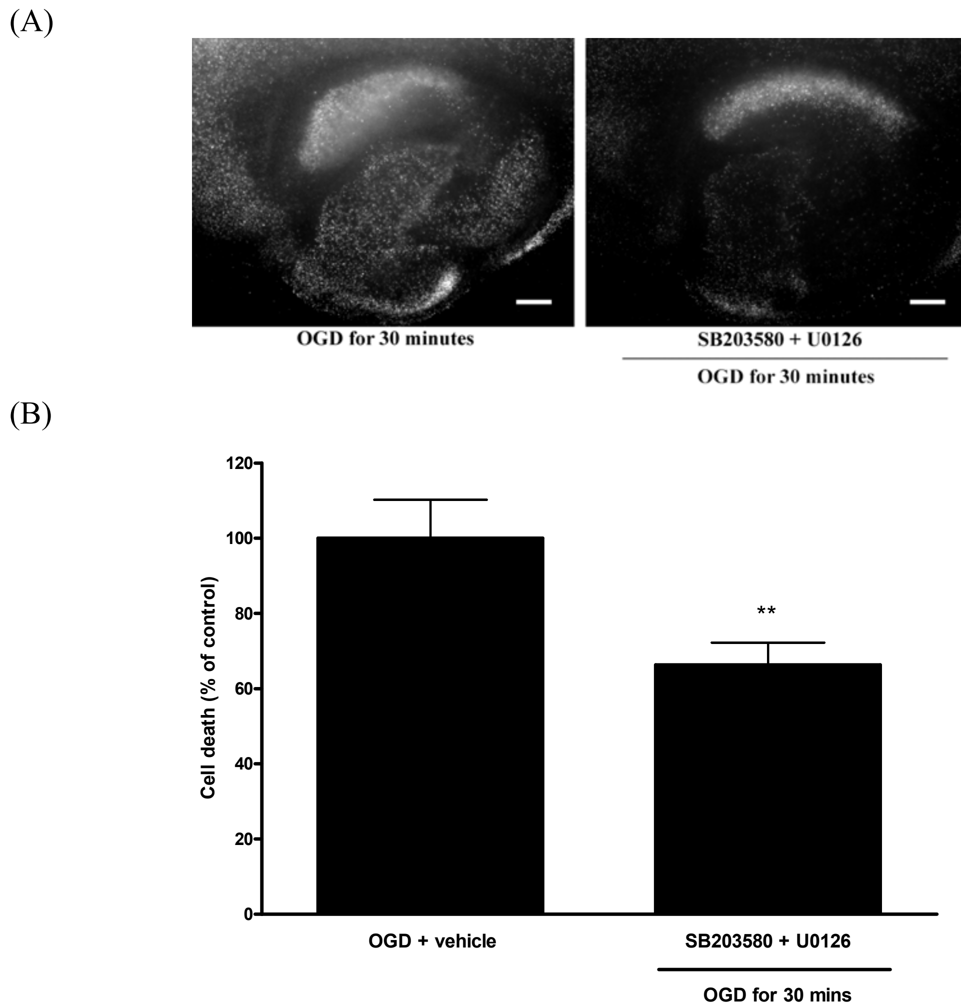


Figure 5.5 Pharmacological inhibition of MAP kinases inhibit OGD-induced toxicity.

Slices were subjected to OGD for 30 minutes and incubated in fresh culture media containing MAP kinase inhibitors with PI (2 μM). The PI images were visualised using epi-inverted fluorescent microscope. (A) Representative PI fluorescence images showing cell death in slices exposed to OGD for 30 minutes and incubated in fresh culture media containing either vehicle or MAP kinase inhibitors for 18 hours. (B) Summary illustrating MAP kinase inhibitors reduce cell death induced by OGD. All experiments were carried out in at least 4 separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. $^{**}p < 0.01$ versus OGD 30 minutes + vehicle. Scale bar: 200 μm.

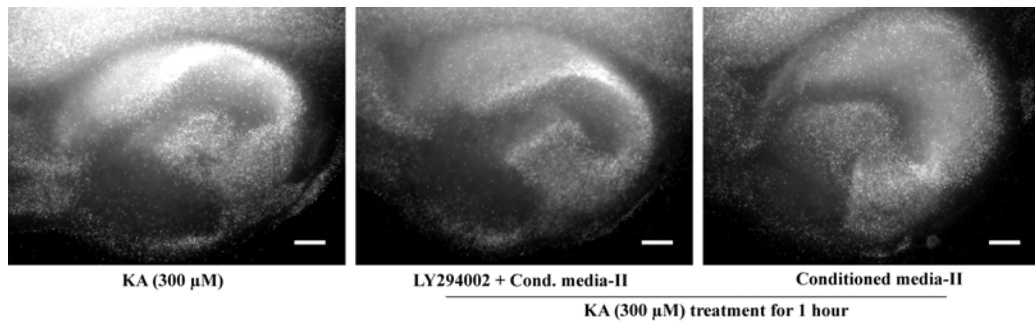
5.2.4 PI-3 kinase pathway is not involved in the observed neuroprotection

Having established the MAP kinase signalling pathways partially underlie the observed neuroprotection, I assessed the involvement of PI-3 kinase in the observed neuroprotection using a pharmacological approach. The involvement of PI-3 kinase was determined by using a PI-3 kinase inhibitor, LY294002 (10 μM) as described previously (Wu *et al.,* 2004; Arai and Lo, 2010). Initially, I investigated whether LY294002 can modulate the KA-induced cell death alone. OSCs were treated with KA (300 μM, 1 hr) in the presence of PI-3 kinase inhibitor and incubated in fresh culture media with PI (2 μM) in the presence of PI-3 kinase inhibitor. There was no alteration in KA-induced cell death (102.9 ± 2.4 % of the KA 300 μM control; $n = 6$ slices from 6 animals). To assess the role of PI-3 kinase in KA-induced cell death, firstly, slices were treated with KA in the presence of PI-3 kinase inhibitor and transferred into conditioned media-II containing LY294002 with PI for 18 hours incubation. Those slices were compared with control slices (slices treated with KA and incubated in conditioned media-II alone for 18 hours). The conditioned media with PI-3 kinase inhibitor exhibited neuroprotection (84.7 ± 3.2 % of the KA 300 μM control; $n = 13$ slices from 6 animals; $p < 0.05$; Figure 5.6) against KA-induced cell death which was similar to the response observed in the conditioned media alone (79.7 ± 3.0 % of the KA 300 μM control; $n = 15$ slices from 6 animals; $p < 0.01$). No differences were found in the level of neuroprotection between conditioned media alone and conditioned media with PI-3 kinase inhibitor suggesting that PI-3 kinase signalling pathway does not underlie the neuroprotection mediated by the conditioned media.

Having determined that PI-3 kinase was not involved in the observed neuroprotection in the KA model, I also assessed the role of PI-3 kinase pathway in the OGD model. OSCs following exposure to OGD for 30 minutes in the presence of PI-3 kinase inhibitor were incubated in conditioned media-II with PI-3 kinase inhibitor for 18 hours and compared with those slices which were incubated in conditioned media-II alone. Consistent with

the KA-induced cell death, the conditioned media in the presence of PI-3 kinase inhibitor was neuroprotective against OGD-induced cell death (72.4 ± 1.4 % of the 30 mins OGD control; n = 6 slices from 4 animals; $p < 0.05$; Figure 5.7) which was similar to the conditioned media alone (65.9 ± 6.0 % of the 30 mins OGD control; n = 4 slices from 3 animals; $p < 0.05$). Thus, this finding suggests that in this model also, PI-3 kinase has no role in the neuroprotection mediated by the conditioned media.

(A)



(B)

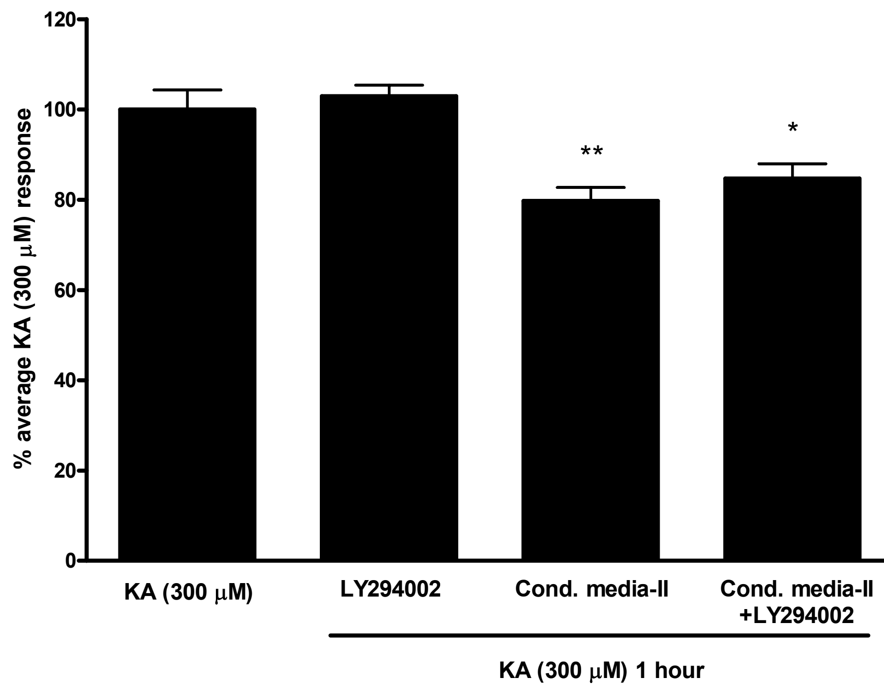


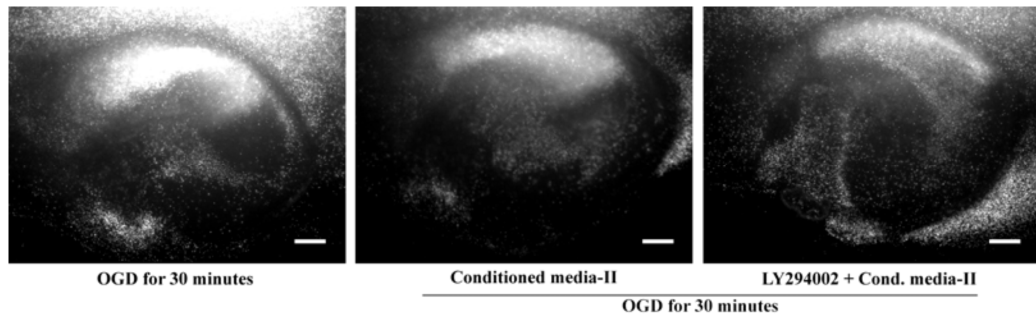
Figure 5.6 PI-3 kinase is not involved in the neuroprotection mediated by conditioned media from lymphocytes in the KA model.

Continued…

Figure 5.6 PI-3 kinase is not involved in the neuroprotection mediated by conditioned media from lymphocytes in the KA model.

Slices were treated with KA (300 μM, 1 hr) in the presence of LY294002 (10 μM), a PI-3 kinase inhibitor and incubated in conditioned media containing PI-3 kinase inhibitors with PI (2 μM) for 18 hours. The PI images were visualised using fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in OSCs following KA treatment either in the presence or absence of PI-3 kinase inhibitor and incubated in conditioned media for 18 hours either with or without PI-3 kinase inhibitor. Control slices were treated with KA and incubated in either fresh culture media or conditioned media-II. (B) Bar chart showing the neuroprotection by the conditioned media in the presence of LY294002. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus KA 300 μM. Scale bar: 200 μm

(A)



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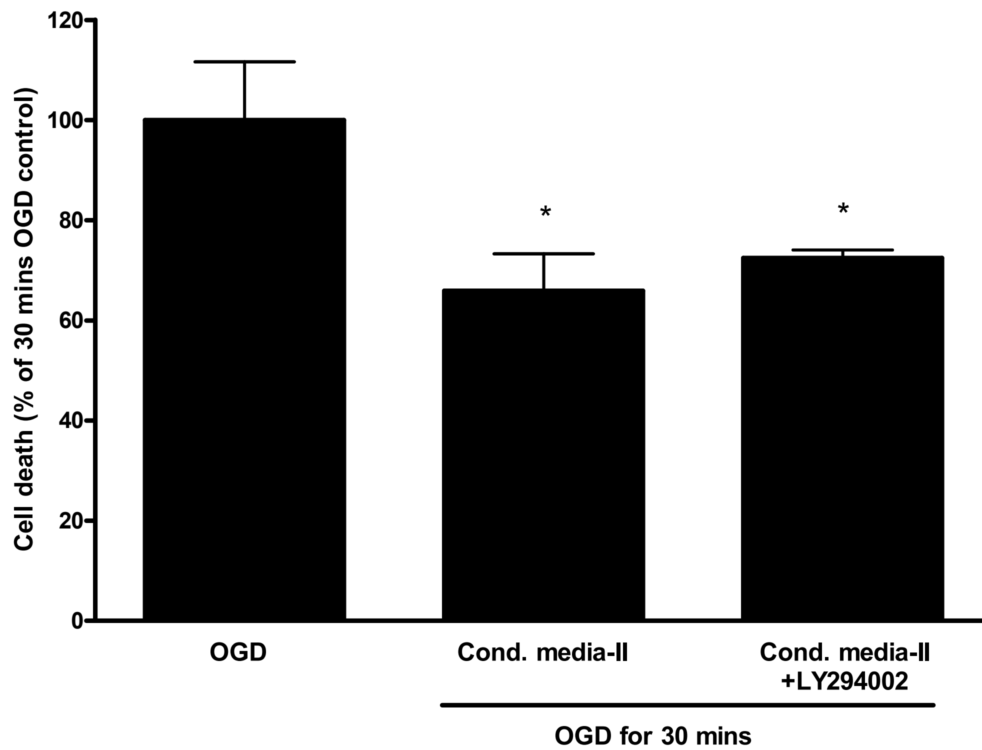


Figure 5.7 The neuroprotection mediated by conditioned media from lymphocytes does not involve PI-3 kinase signalling pathway in the OGD model.

Continued…

Figure 5.7 The neuroprotection mediated by conditioned media from lymphocytes does not involve PI-3 kinase signalling pathway in the OGD model.

Slices were subjected to OGD for 30 minutes in the presence of LY294002 (10 μM), a PI-3 kinase inhibitor and incubated in conditioned media containing PI-3 kinase inhibitors with PI (2 μM) for 18 hours. The PI images were visualised using fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices following exposure for 30 minutes to OGD either in the presence or absence of PI-3 kinase were incubated in the conditioned media for 18 hours either with or without PI-3 kinase inhibitor. Control slices were subjected to OGD for 30 minutes and incubated in fresh culture media. (B) Bar chart showing the neuroprotection against OGD-induced cell death by conditioned media either in the presence or absence of PI-3 kinase inhibitor. All experiments were carried out in at least 3 separate animal preparations. Data presented as mean ± SEM. $*p < 0.05$ versus OGD 30 minutes. Scale bar: 200 μm.

5.3 Discussion

It is now evident that astrocytes play a key role during neuroinflammation (Brambilla *et al.*, 2005; van Loo *et al.*, 2006). Here, I show that the inhibition of astrocytic function using FAc, a glial cell metabolic inhibitor that has been used previously to investigate the role of astrocytic function (Swanson and Graham, 1994; Shigetomi *et al.*, 2008; Greenwood and Bushell, 2010; Henneberger *et al.*, 2010) abolished the lymphocyte-mediated neuroprotection in KA-induced cell death which suggests that the observed neuroprotection is at least partially mediated via astrocytes. Supporting the current finding, several studies have reported a neuroprotective role for astrocytes (Brown, 1999; Sofroniew, 2005; Myer *et al.*, 2006). Astrocytes normally contribute to homeostatic maintenance including ions, water and neurotransmitters in the CNS (Anderson and Swanson, 2000; Matsuura *et al.*, 2002; Sofroniew and Vinters, 2010; Allaman *et al.*, 2011) and are able to clear excess glutamate from the synaptic cleft (Danbolt, 2001; Gadea and López-Colomé, 2001) since high extracellular level of glutamate is neurotoxic (Brown, 1999). It has been reported that astrocytes stimulated by T cell-derived glutamate protect neurons from oxidative stress (Garg *et al.*, 2008). Garg *et al.* (2008) have shown that T cells produce more glutamate after activation and this signal causes metabolic modification, leading to an astrocyte-mediated neuroprotective effects. Glutamate uptake by astrocytes has been correlated with a decrease in extracellular cystine and increase in glutathione synthesis by astrocytes (Reichelt *et al.*, 1997; Rimaniol *et al.*, 2000; Lewerenz *et al.*, 2006; Shih *et al.*, 2006) that can protect neurons from oxidative stress. In addition, glutamate clearance capacity of astrocytes is enhanced by T cell-derived cytokines such as IFN-γ and IL-2 (Garg *et al.*, 2008). Moreover, astrocytes have been suggested to be involved in attenuation of microglial function (Vincent *et al.*, 1997; Hailer *et al.*, 2001; Min *et al.*, 2006) thus limiting the microglial mediated injury since microglia on activation release pro-inflammatory cytokines and reactive oxygen species which have negative role in neuronal survival (Block *et al.*, 2007; Belanger and Magistretti, 2009). Moreover, astrocytes release various neurotrophic factors including neurotrophic growth factor (NGF), BDNF, glial-

cell lined derived neurotrophic factors (GDNF), IGF and TGF-β (Blondel *et al.*, 2000; Hailer *et al.*, 2001; Farina *et al.*, 2007) along with antioxidants including glutathione and ascorbic acid that can contribute to neuroprotection against oxidative stress (Wilson, 1997; Dringen, 2000; Vargas *et al.*, 2008). However, my data showed that FAc alone was not toxic to slices and also did not induce more cell death in KA treated OSCs. This finding suggests that astrocytes are not directly involved in the modulation of KA-induced cell death but acting as a mediator in the observed neuroprotection.

Notably, in OGD-induced cell death, the inhibition of astrocytic function does not have an effect on the lymphocyte-mediated neuroprotection, which is contrast to the finding from KA-induced cell death. A possible explanation for this inconsistent result might be due to the different cell targets in OGD-induced cell death and KA-induced cell death. In stroke, not only neurons but other non-neuronal cells including astrocytes, microglia and endothelial cells are affected by ischemic injury (Doyle *et al.*, 2008). It has been shown that OGD induces astrocytic death in astrocytes culture (Cao *et al.*, 2010). On the other hand, in KA-induced cell death, the majority of cells affected by KA are neurons (Schousboe *et al.*, 1992; Cheng and Sun, 1994) and it has also been reported that astrocytes were not affected by 24 hours incubation with various concentrations (0.01 to 2 mM) of KA (Waniewski and McFarland, 1990). Therefore, it is likely that OGD might induce astrocytic death or lowered metabolic activities of astrcoytes under the present experimental conditions and the application of a glial cell metabolic inhibitor may not have any effect. Taken together, these findings suggest that neuroprotection against OGD-induced cell death is mediated by soluble mediators released by lymphocytes and astrocytes are not involved.

A role of microglia in the observed neuroprotection cannot be excluded as microglial cells have also been implicated in neuroprotection (Toku *et al.*, 1998; Butovsky *et al.*, 2005; Shaked *et al.*, 2005; Polazzi and Monti, 2010). Microglia exhibit neuroprotection by clearing cell debris (Napoli and Neumann, 2009), engulfment of neutrophils (Neumann *et al.*, 2008) and release of neuroprotective factors including BDNF, IGF-1

and TGF-β (Kiefer *et al.*, 1995; Lalancette-He´bert *et al.*, 2007; Madinier *et al.*, 2009). An *in vitro* study has shown that microglial cells protect neurons from nitric-oxide induced neuronal injury (Toku *et al.*, 1998) but the mechanism of this neuroprotection is still unknown. It has been suggested that microglial cells stimulated by INF-γ and IL-4 are neuroprotective (Butovsky *et al.*, 2005; Shaked *et al.*, 2005). However, I could not assess the role of microglia in the present study due to the lack of specific pharmacological agents to inhibit microglial function. Although, minocycline has been widely used to modulate the activity of microglia (Du *et al.*, 2001; Lin *et al.*, 2001; Wu *et al.*, 2011), minocycline has also been suggested to modulate astrocytic activity (Yrjänheikki *et al.*, 1998; Du *et al.*, 2001). Recently, FTY720 (Fingolimod), the first FDA approved oral drug for MS, has also been used to investigate its effect on microglial activity which showed apoptosis of microglia (Yoshino *et al.*, 2011). However, its effect on astrocytes has also been demonstrated (Osinde *et al.*, 2007; Choi *et al.*, 2011). Therefore, this is an important issue for future research to examine by manipulating microglial function without affecting astrocytic function.

As described earlier, little attention has been paid to the underlying cellular signalling mechanism(s) of lymphocyte-mediated neuroprotection, I investigated signalling pathways that are involved in normal cellular functions, including cell differentiation, growth, cell division, survival and cell death (Rameh and Cantley, 1999; Chang and Karin, 2001; Dong *et al.*, 2002; Foster *et al.*, 2003) to determine whether these pathways underlie the lymphocyte-mediated neuroprotection.

First, I investigated MAP kinase signalling pathways which indicated that neuroprotection may be mediated via the inhibition of ERK 1/2 and p38 MAP kinase activity without altering the effect of JNK 1/2. I showed for the first time that the MAP kinase signalling pathways are involved in the lymphocyte-mediated neuroprotection. Similar findings have been previously demonstrated by our laboratory but in PAR-2 mediated neuroprotection against KA-induced cell death in rat OSCs (Greenwood and Bushell, 2010). Normally, ERK activation is necessary for cell proliferation, cell

differentiation and cell growth whereas JNK 1/2 and p38 MAP kinase are activated by stress (Xia *et al.*, 1995; English *et al.*, 1999; Chang and Karin, 2001; Dong *et al.*, 2002). It has been shown that ERKs contribute in neuronal survival by inhibiting JNK/p38 MAP kinases (Xia *et al.*, 1995) and the ERK activation was suggested to be required in BDNF-mediated neuroprotection against hypoxic-ischemic injury as well as oxidative glutamate toxicity (Han and Holtzman, 2000; Rössler *et al.*, 2004). Further, it has also been demonstrated that activation of ERKs was responsible for neuroprotection in neuronal culture against cytosine arabinoside (araC)-induced toxicity (Xue *et al.*, 2000). In contrast to these findings, the present data has shown that the observed neuroprotection is mediated via the down-regulation of ERK under the present experimental conditions. A possible explanation for this variation might be due to different experimental models being used. Previous studies did not use lymphocytes and therefore different result might be expected. Another explanation might be due to the heterogeneous cell population of OSCs and it is noteworthy that OSCs contain mainly astrocytes and microglia along with neurons. Therefore, it can be suggested that the down-regulation of ERKs observed in the present study is a combined response of neuronal and non-neuronal cells and the response in pure cell preparations may vary. Meanwhile, a pharmacological approach to inhibit an ERK inhibitor and a p38 inhibitor to mimic lymphocyte-induced reduction in MAP kinase activity demonstrated the neuroprotection against KA-induced cell death as well as against OGD induced cell death. This finding suggests that lymphocyte mediated reduction of MAP kinase activity can play a crucial role in the observed neuroprotection. Supporting the present finding, several studies have demonstrated that inhibition of ERK activity can be neuroprotective (Alessandrini *et al.*, 1999; Satoh *et al.*, 2000; Namura *et al.*, 2001; Maddahi and Edvinsson 2010) since persistent activation of ERKs has been found to induce apoptosis (Chen *et al.*, 2005; Tong *et al.*, 2011). The administration of an ERK inhibitor in a stroke model of rodents significantly reduced the infract volume size thus reducing the brain damage (Alessandrini *et al.*, 1999; Maddahi and Edvinsson, 2010). One of the common mechanism of cell death in neurodegenerative diseases is oxidative stress mediated via reactive oxygen species (Pettmann and Henderson, 1998) and the accumulation of

reactive oxygen species has been associated with delayed and persistent ERK activation (Stanciu *et al.*, 2000). Stanciu *et al.* (2000) have further demonstrated that the persistent activation of ERK activity can be correlated with the glutamate-induced toxicity in neuronal cell line and cortical neuronal cultures which was successfully reversed by U0126, an ERK inhibitor. Recently, it has also been shown that the persistent activation of ERK in the brain of KA treated mice (De Lemos *et al.*, 2010) while zinc-induced cell death in a neuronal cell line has been suggested to be mediated via the activation of ERK (Seo *et al.*, 2001). Moreover, brain samples from stroke patients showed the upregulation of ERK activity (Slevin *et al.*, 2000) and it has also been shown that an upregulation of ERK activity after ischaemia and reperfusion contributes to cell death in an animal model of stroke which was attenuated by an ERK inhibitor (Alessandrini *et al.*, 1999; Namura *et al.*, 2001). These findings therefore suggest that inhibition of ERK activity contributes neuroprotection under pathological conditions and recently have been a target for therapy in neurodegeneration (Cheung and Slack, 2004; Yu *et al.*, 2010). On the other hand, p38 MAP kinase activity has been reported to be activated in various neurodegenerative diseases including PD and stroke (Du *et al.*, 2001; Lin *et al.*, 2001; Nozaki *et al.*, 2001) and down-regulation of this pathway is considered to be a novel therapy in these diseases (Harper and Wilkie, 2003; Yasuda *et al.*, 2011). The activation of p38 MAP kinases after withdrawal of growth factors in neuronal cultures has been neurotoxic which was prevented by a p38 MAP kinase inhibitor (Heidenreich and Kummer, 1996; Horstmann *et al.*, 1998). This suggests that the activation of p38 MAP kinase is neurotoxic. In addition, in an animal model of AD, the accumulation of tau proteins and release of pro-inflammatory mediators have been correlated with the activation of p38 MAP kinase leading to cell death (Savage *et al.*, 2002; Culbert *et al.*, 2006). Taken together, the present data support that down-regulation of p38 MAP kinase contributes to neuroprotection. Although, there is evidence for the involvement of JNK signalling pathways in various neurodegenerative diseases including PD and stroke contributing to cell death (Kuan and Burke, 2005; Benakis *et al.*, 2010), no alteration in JNK activity has been observed under the present experimental conditions. However, the role of JNK3 in the observed neuroprotection cannot be excluded as JNK3 has been

suggested to be widely distributed in the CNS (Mohit *et al.*, 1995) and JNK3 deficiency reduces neurotrophic factors withdrawal mediated cell death in neuronal cultures (Bruckner *et al.*, 2001). Several studies have also shown the role of JNK3 in neurodegeneration (Yang *et al.*, 1997; Morishima *et al.*, 2001; Hunot *et al.*, 2004). However, the current study was unable to investigate the role of JNK3 under the present experimental conditions due to a lack of specific antibody for JNK3 and a further study with more focus on JNK3 is therefore suggested.

As described earlier, PI-3 kinase is involved in the regulation of cellular functions including cellular differentiation, growth, proliferation, survival, intracellular trafficking and glucose homeostasis (Fry, 1994; Rameh and Cantley, 1999; Foster *et al.*, 2003). Therefore along with MAP kinase signalling pathways, next I investigated the role of PI-3 kinases in the lymphocyte-mediated neuroprotection under the present experimental conditions. Interestingly, I showed that PI-3 kinase does not appear to play a role in neuroprotection mediated by conditioned media from lymphocytes in KA-induced or OGD-induced cell death. Normally, activation of PI-3 kinase is associated with BDNF/neurotrophins-mediated neurite growth and neuritogenesis (Patapoutian and Reichardt, 2001; Tang, 2003) and several studies have demonstrated the neuroprotective role of PI-3 in an *in vitro* model for stroke and in neuronal cultures (Wu *et al.*, 2004; Zamin *et al.*, 2006; Sun *et al.*, 2010). Further, it has been demonstrated that the loss of dopaminergic neurons in an animal model of PD was associated with a defect in PI-3 kinase mediated signalling pathways. However, my findings are not consistent with these previous studies. A possible explanation for this inconsistency might be due the different approaches being taken by previous studies. The previous studies which showed the involvement of PI-3 kinase in neuroprotection were more focused on specific molecules like estrogen derivative (Dhandapani *et al.*, 2005) or IGF-1 (Sun *et al.*, 2010) or specific drugs such as resveratrol (Zamin *et al.*, 2006) whereas lymphocytes have been used in the present study. It is noteworthy that lymphocytes release various cytokines, chemokines and growth factors (Abbas *et al.*, 1996; Kerschensteiner *et al.*, 1999; Moalem *et al.*, 2000; Wan and Flavell, 2009) and these mediators can activate or

inhibit various signalling pathways (Han and Holtzman, 2000; Zhu *et al.*, 2002; Culbert *et al.*, 2006; Lisak *et al.*, 2011). When taken together, it can be suggested that multiple signalling pathways might have been involved in the observed neuroprotection under the present experimental conditions. In addition, PI-3 kinase has also been suggested to be a key modulator in inflammatory responses (Wymann *et al.*, 2003; Haylock-Jacobs *et al.*, 2011) and has been a potential target for a drug development (Stein and Waterfield, 2000). It has been demonstrated that PI-3 kinase deficiency slows the progression of disease and improves the clinical outcomes in EAE induced mice (Berod *et al.*, 2011). However, there are different types of PI-3 kinases and their specific functions are still under investigation (Maffucci *et al.*, 2005; Williams *et al.*, 2009). Thus, more focussed research on their specific types can be suggested to investigate their precise role in lymphocyte-mediated neuroprotection.

In summary, the data in this chapter show that astrocytes play a critical role in KA-induced cell death, but that this may not be the case in OGD-induced cell death, although involvement of other cells (including microglia) cannot be excluded. To the best of my knowledge this is the first time that inhibition of MAP kinases have been implicated to underlie lymphocyte-mediated neuroprotection which was further confirmed using a pharmacological approach. In addition, the current study has shown that the PI-3 kinase signalling pathway is not involved in the neuroprotection mediated by conditioned media from lymphocytes in both KA and OGD induced cell death.. Therefore, the evidence from this study suggests that astrocytes can modulate the neuroprotective response of lymphocytes depending on the CNS pathology and the MAP kinase signalling pathways, but not the PI-3 kinase signalling pathway, underlie lymphocyte-mediated neuroprotection.

6 Role of lymphocytes in the cerebral cortex

In the previous chapter I investigated the role of lymphocytes in an *in vitro* model of neurodegenerative diseases in the hippocampus. As a section of cortex was also present in the OSC along with the hippocampus (Figure 6.1), I investigated the effect of lymphocytes on induced-neurotoxicity in the cortical region.

6.1 Introduction

As described previously (see section 1.1), the cerebral cortex has various regions having distinct functions and is a major part of the CNS. The cerebral cortex is affected by various neurodegenerative including AD, MS and PD (Olanow and Tatton, 1999; Akiyama *et al.*, 2000; Bö *et al.*, 2006) and suggested to be the more affected region during AD characterised by the accumulation of Aβ proteins and increase levels of various inflammatory mediators such as IL-6, TNF-α and cox-2 (Wood *et al.*, 1993; Watanabe *et al.*, 1996; Akiyama *et al.*, 2000). Similarly, early neurodegeneration has been seen in cerebral cortex of MS and PD patients (MacDonald and Halliday, 2002; Bö *et al.*, 2003; Bö *et al.*, 2006; Obeso *et al.*, 2010) which suggest that the cerebral cortex is an early site for the pathology. Moreover, it is also evident that cerebral cortex is affected by stroke (Hossmann, 2006; Offner *et al.*, 2006; McColl *et al.*, 2009; Iadecola and Anrather, 2011). In addition, the infiltration of lymphocytes during neurodegenerative diseases is well established (Rezai-Zadeh *et al.*, 2009; Chung *et al.*, 2010; Fumagalli *et al.*, 2011) and the neurotoxic and neuroprotective role of lymphocytes in various disease models have been extensively discussed (Hammarberg *et al.*, 2000; Byram *et al.*, 2004; Frenkel *et al.*, 2005; Chiu *et al.*, 2008). It has been suggested that the infiltration of T cells into the CNS induces neurodegeneration following traumatic brain injury (Fee *et al.*, 2003) as well as in a mouse model of PD (Brochard *et al.*, 2009). In contrast, the neuroprotective role of T cells has also been demonstrated in a mouse model of PD (Reynolds *et al.*, 2007). Further, T cells have been shown to be involved in glial cell mediated neuroprotection and also delaying the

disease progression in a mouse model of ALS (Beers *et al.*, 2008; Chiu *et al.*, 2008). However, there is a lack of understanding regarding any differences in the response of the cortical region and hippocampal region to lymphocytes in neurodegenerative diseases. Therefore, I have investigated the role of lymphocytes in the cortical region using two models of neurodegenerative diseases: KA-induced cell death and OGD-induced cell death. Similar to the hippocampus, I also investigated the involvement of astrocytes as well as the underlying cellular signalling mechanism(s) using a pharmacological approach.

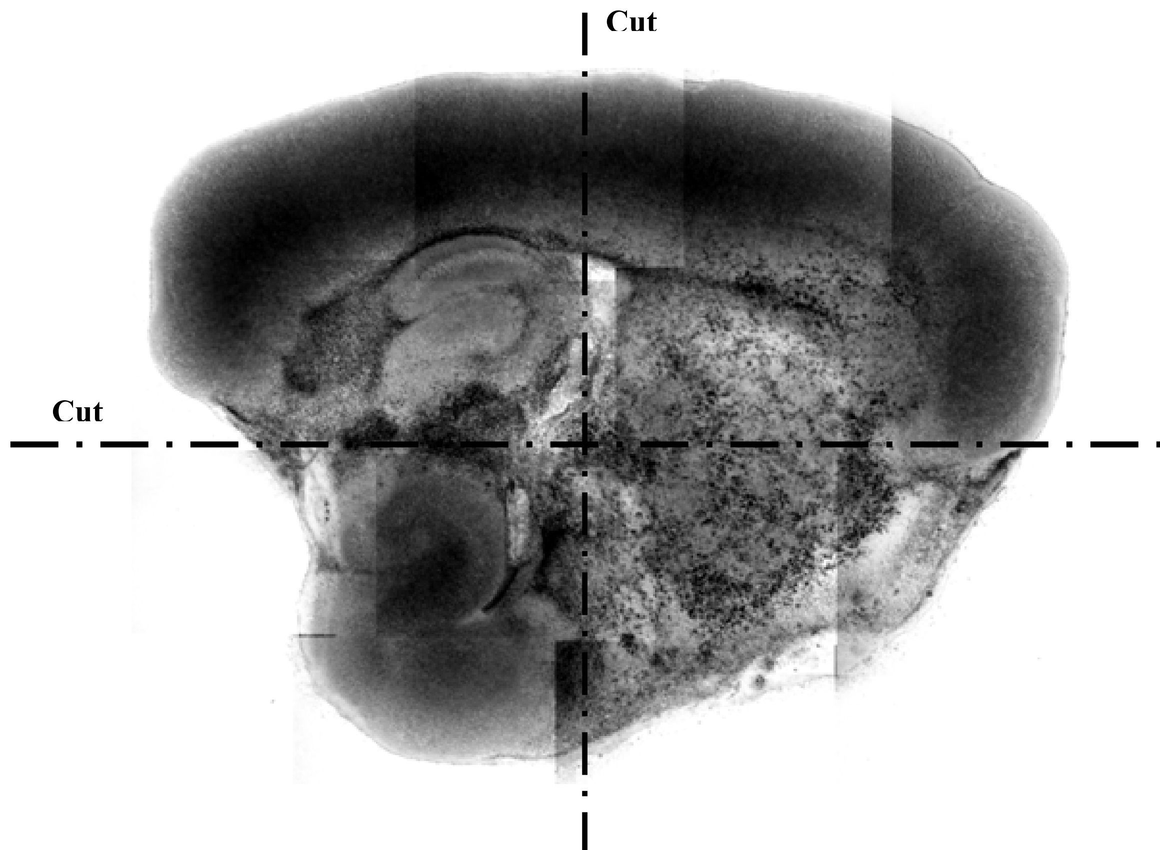


Figure 6.1 Composite image of a saggittal section of organotypic brain slice.

Brain slices of 300 μm were prepared using mouse pups (4-6 DIV) and hippocampus along with cerebral cortex was cut off from the whole saggittal section. These slices were then incubated in serum-containing preparation media on first day and subsequently replaced by serum-free culture media next day and serum-free culture media was continued for rest of the culture period.

6.2 Results

6.2.1 KA-induced neurotoxicity in OSCs is concentration dependent

Having established that KA-induced cell death in the hippocampus is concentration dependent, I also investigated the response of the cortical region to KA. Similar to the hippocampus, OSCs were treated with five different concentrations of kainate (KA, 3 μM - 3 mM) to assess the neuronal death pattern. Slices were treated with KA for 1 hour, transferred to fresh culture media containing PI (2 μM) and then incubated for 18 hours.

KA induces neurotoxicity in a concentration dependent manner with a concentration of 3 mM KA inducing maximum neuronal death. All data are expressed in average mean fluorescent intensity, more the fluorescent intensity, more number of cell death. The concentration-dependent manner of neuronal death can be also seen in representative fluorescence images (Figure 6.2 A). The avergae mean fluorescent intensity for 300 μM KA was 50.6 ± 5.1 (n = 35 from 22 animals) while for 3 mM KA (n = 15 slices from 5 animals) the avergae mean fluorescent was found to be 69.1 ± 3.2. From the concentration-response curve, the effective concentration for 50 % cell death (EC_{50}) for KA was found to be 152.0 ± 1.1 μM (Figure 6.2 B). The concentration of 300 μM KA was used as a positive control for the rest of my study in this thesis as 300 μM KA didn't induce maximum cell death. The cell death induced by KA (300 μM) was set to 100% and all other values were compared with this concentration.

To clarify that the KA-induced neurotoxicity was mediated via ionotropic glutamatergic receptors as has been reported by previous studies (Wang *et al.*, 2005; Greenwood and Bushell, 2010; Contractor *et al.*, 2011), experiments were performed in the presence of NBQX (20 μM), antagonist for AMPA/KA and DL-AP5 (100 μM), NMDA antagonist. I show that the neurotoxicity induced by KA (300 μM) was completely blocked by NBQX and DL-AP5 (5.1 ± 2.7 % of the 300 μM KA control, n = 6 slices from 4 animals; $p < 0.001$; Figure 6).

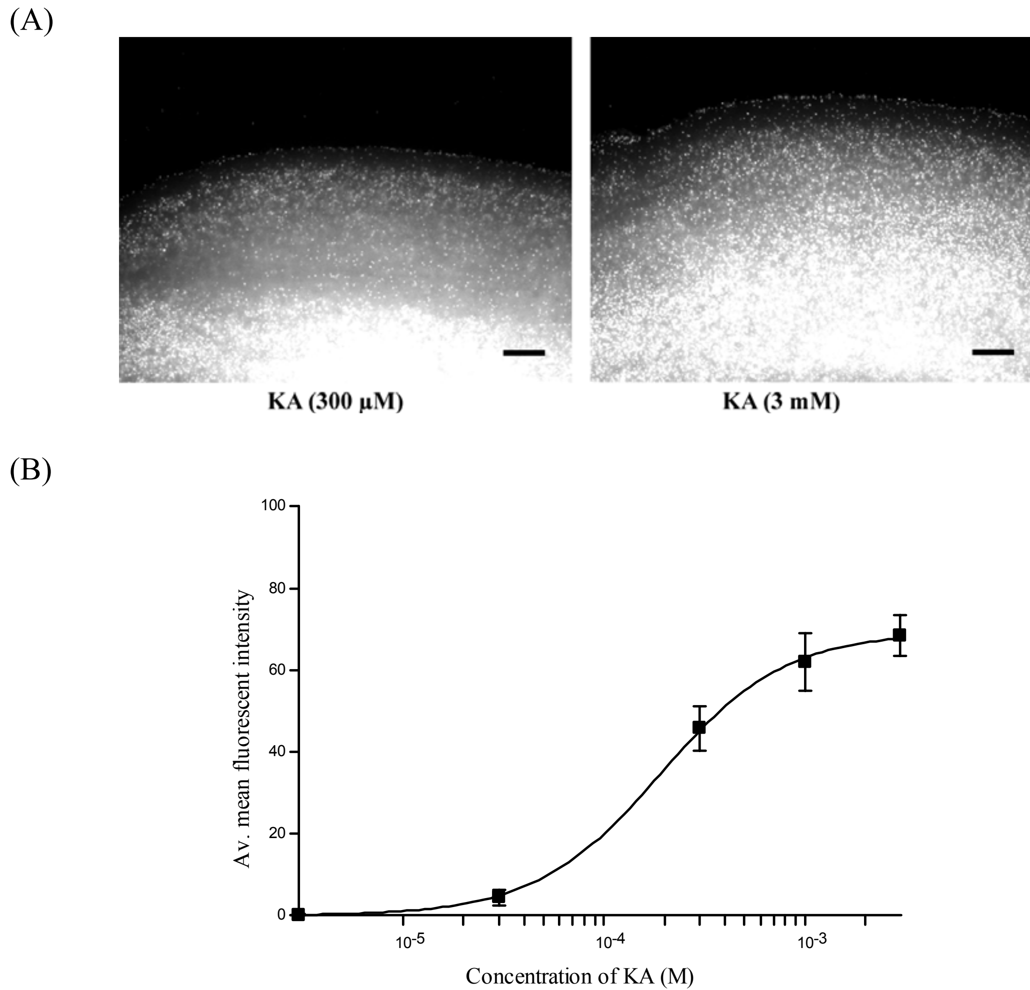


Figure 6.2 KA induces neuronal death in a concentration-dependent manner.

(A) Representative PI images showing cell death induced in slices treated with KA (300 μM) or KA (3 mM) for 1 hour and incubated in the fresh culture media for 18 hours. (B) Concentration response curve showing cell death induced by KA. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. Scale bar: 200 μm

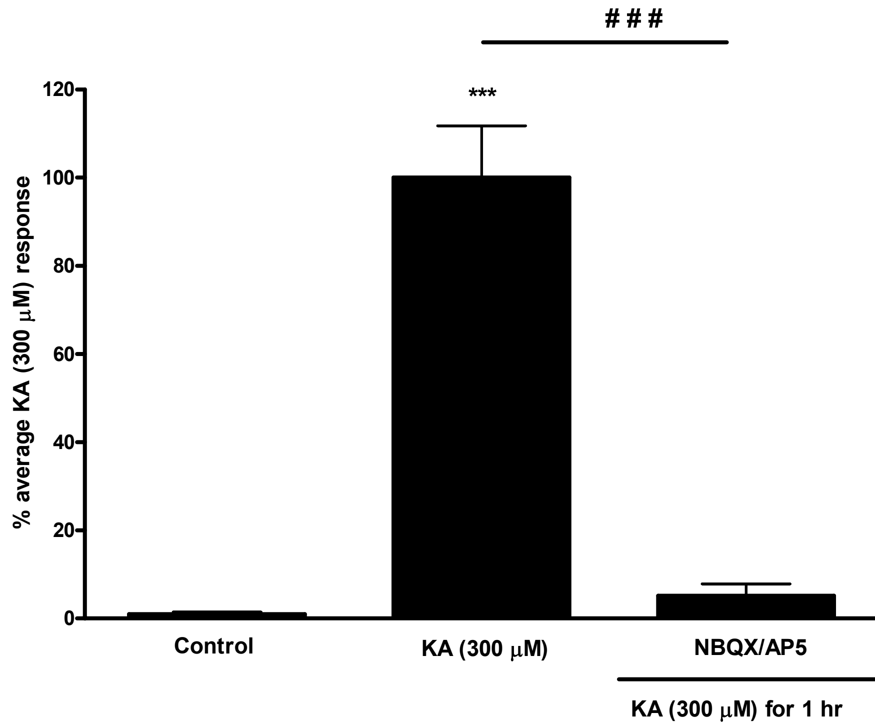


Figure 6.3 KA-induced cell death is mediated via ionotropic glutamatergic receptors.

Bar chart showing the effect of NBQX, an antagonist for AMPA/KA receptor and AP5, an antagonist of NMDA receptor against KA-induced cell death. Slices treated with KA (300 μM) for 1 hour in the presence or absence of NBQX and AP5 and incubated in fresh culture media for 18 hours reduced KA-induced cell death. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. ***p < 0.001 versus fresh culture media (control); ###p < 0.001 versus NBQX/AP-5 treated slice.

6.2.2 Lymphocytes do not induce toxicity in cortex of OSCs under normal culture conditions

Consistent with the hippocampus, lymphocytes do not induce toxicity in the cortex of OSCs under normal culture conditions (Figure 6.4). Slices were co-cultured with lymphocyte preparations containing either non-activated or activated T cells for 18 hours and compared with slices which were treated with 300 μM KA for 1 hour and incubated in fresh culture media with PI (2 μM) for 18 hours. Neither lymphocytes with non-activated T cells (7.8 ± 3.3 % of the 300 μM KA control; n = 17 slices from 6 animals; $p<0.001$) nor lymphocytes with activated T cells (6.3 ± 3.2 % of the 300 μM KA control; n = 9 slices from 4 animals; $p<0.001$) were toxic to OSCs under normal culture conditions.

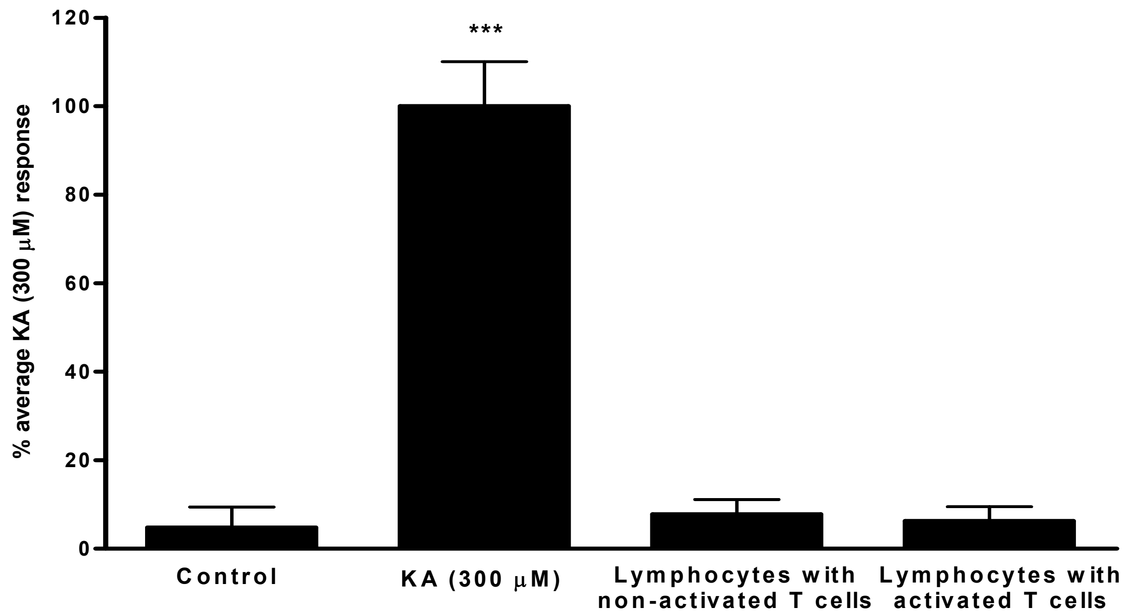


Figure 6.4 Lymphocyte preparations are not harmful to cortex of OSCs under normal culture condition.

Lymphocyte preparations containing either non-activated T cells or activated T cells do not induce toxicity in OSCs when co-cultured for 18 hours. Toxicity is compared to slices treated with 300 μM KA for 1 hour and incubated in fresh culture media for 18 hours. All experiments were carried out in at least 4 separate animal preparations. Data presented as mean ± SEM. ***$p < 0.001$ versus fresh culture media (control).

6.2.3 Lymphocyte preparations containing either non-activated T cells or activated T cells are neuroprotective against both KA and OGD induced cell death.

Having established that lymphocytes do not induce toxicity to cortex of OSCs under normal culture condition, I investigated whether lymphocytes in the presence of KA can modulate KA-induced toxicity. For this purpose, slices were co-cultured with lymphocyte preparations containing non-activated T cells in the presence of KA (300 μM) for 1 hour and incubated in fresh culture media with PI (2 μM) for 18 hours. Quantification of PI uptake showed 135.3 ± 14.8 % of cell death (n =15 slices from 8 animals; $p > 0.05$ versus KA 300 μM; Figure 6.5) which suggest that lymphocytes cannot modulate KA-induced toxicity at that time point. Furthermore, as several studies have shown either neurotoxic (Brochard *et al.*, 2009; Huang *et al.*, 2009) or neuroprotective (Moalem *et al.*, 1999; Beers *et al.*, 2008) role of activated T cells, T cells within the lymphocyte preparations were activated using anti-CD3 (see section 2.2.2.3) to investigate their role under the present experimental conditions. Similar to the previous experimental condition, after treating slices with KA for 1 hour in the presence of lymphocyte preparation containing activated T cells, slices were incubated in fresh culture media with PI for 18 hours and quantified cell death. Lymphocyte preparations containing activated T cells exhibited 113.6 ± 10.5 % of cell death (n = 14 slices from 6 animals; $p > 0.05$ versus KA 300 μM) when compared with 300 μM KA and no significant differences were found in the level of response between lymphocyte preparations containing non-activated T cells and lymphocyte preparations containing activated T cells. This suggests that activation of T cells within the lymphocyte preparations does not modulate KA-induced cell death.

To investigate whether a longer incubation period with lymphocyte preparations can modulate KA-induced toxicity, I therefore modified the model such that slices were treated with KA for 1 hour and co-cultured with lymphocyte preparations containing non-activated T cells for 18 hours. As shown in Figure 6.6, lymphocyte preparations

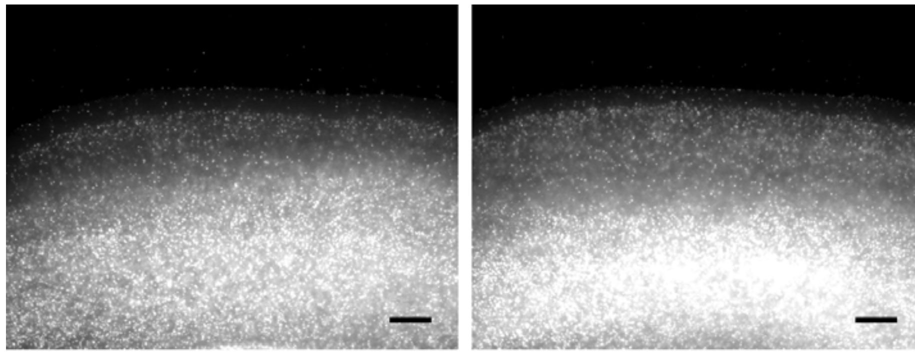
containing non-activated T cells significantly reduced KA-induced cell death (74.9 ± 5.2 % of the 300 μM KA control; n = 48 slices from 18 animals; p < 0.01) which suggests that lymphocytes are neuroprotective. In addition, as the pathogenic response of activated T cells has been demonstrated by various studies (Appel, 2009; Brochard *et al.*, 2009; Huang *et al.*, 2009), I also investigated whether the presence of activated T cells within the lymphocyte preparations have an effect on the observed neuroprotection exhibited by the lymphocyte preparations containing non-activated T cells. Having established in Figure 3.6 that anti-CD3 activated T cells within the lymphocyte preparations do not induce toxicity and lymphocyte preparations containing non-activated T cells are neuroprotective, I treated slices with KA for 1 hour and incubated with lymphocyte preparations containing activated T cells for 18 hours. Lymphocyte preparations containing activated T cells reduced neuronal death against KA-induced toxicity (79.1 ± 4.8 % of the 300 μM KA control; n = 50 slices from 17 animals; p < 0.05). Moreover, no significant differences were found in the level of neuroprotection between lymphocyte preparations containing non-activated T cells and lymphocyte preparations containing activated T cells. Together, these data suggest that lymphocyte preparations are neuroprotective.

Having established that lymphocyte preparations were neuroprotective and majority of cells present in lymphocyte preparations were T cells (see section 4.2.2), I also investigated the role of purified non-activated T cells and purified activated T cells against KA-induced cell death in the cortical region. Slices were treated with KA (300 μM, 1 hour) and incubated with media containing purified non-activated T cells for 18 hours in the presence of PI (2 μM). The purified T cells reduced cell death significantly (72.0 ± 3.8 % of the KA 300 μM control; n = 16 slices from 9 animals; p < 0.01; Figure 6.7) against KA-induced neurotoxicity. Again, to determine whether activation of purified T cells can alter the observed neuroprotection, I also investigated the role of purified activated T cells which were activated by anti-CD3/anti-CD28 (see section 2.2.2.3). Following treatment with KA, slices were incubated with purified activated T cells for 18 hours and assessed PI uptake. As similar to the purified non-activated T

cells, the purified activated T cells also significantly reduced KA-induced neurotoxicity (63.8 ± 8.0 % of the KA 300 μM control; n = 7 slices from 5 animals; $p < 0.01$). In addition, there were no significant differences in the neuroprotective response between purified non-activated T cells and purified activated T cells. These findings suggest that the neuroprotection exhibited by T cells is independent of the activation state of T cells similar to the hippocampus but their neuroprotection is time dependent.

Moreover, I also investigated the role of lymphocyte preparations in OGD-induced cell death. OSCs were subjected to OGD for 30 minutes and co-cultured with lymphocyte preparations with PI for 18 hours. Similar to the effects observed on KA-induced cell death, lymphocyte preparations significantly reduce cell death induced by OGD (55.2 ± 8.3 % of the 30 mins OGD control; n = 16 slices from 4 animals; $p < 0.01$; Figure 6.8).

(A)



(B)

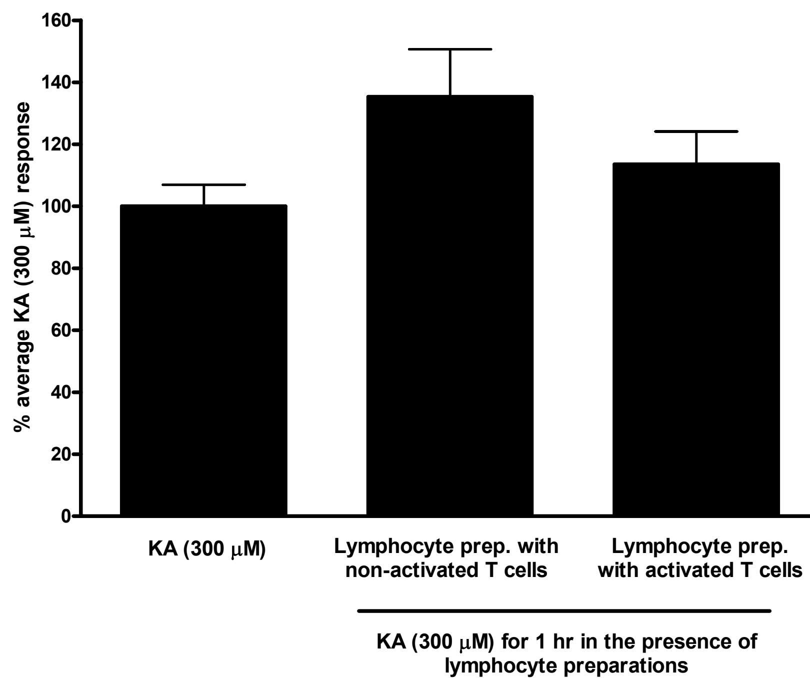


Figure 6.5 Lymphocytes do not alter KA-induced cell death when co-cultured for 1 hour in the presence of KA.

Continued…

Figure 6.5 Lymphocytes do not alter KA-induced cell death when co-cultured for 1 hour in the presence of KA.

Following culturing OSCs for 13-15 days in culture media, they were treated with KA (300 μM, 1 hr) in the presence of $1X10^6$ lymphocytes beneath the insert membrane and incubated in fresh culture media for 18 hours in the presence of PI (2 μM). The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices cultured with or without lymphocyte preparations either containing non-activated or activated T cells in the presence of KA which were then incubated in fresh culture media for 18 hours. (B) Bar chart illustrating the quantification of cell death after slice treated with KA (300 μM) for 1 hour in the presence of lymphocytes and transferred to fresh media for recovery. All experiments were carried out in at least 6 separate animal preparations. Data presented as mean ± SEM. Scale bar: 200 μm.

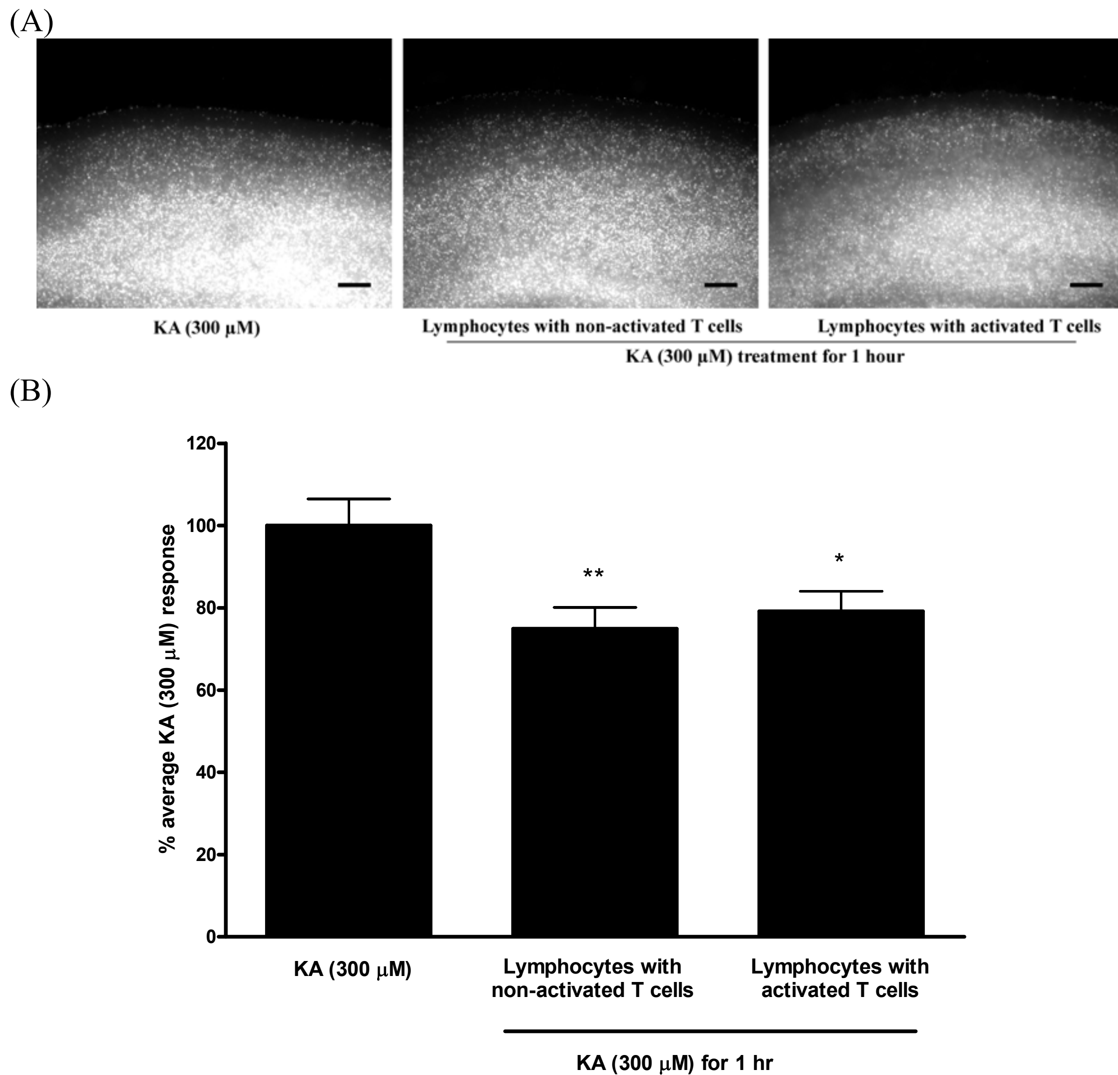
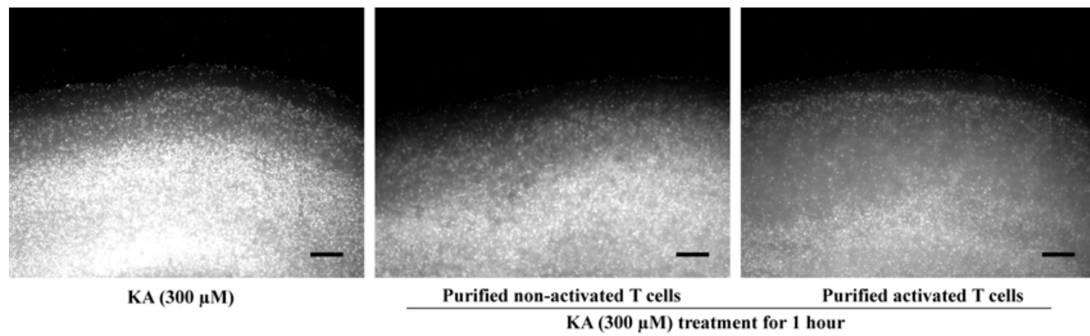


Figure 6.6 Lymphocytes are neuroprotective against KA-induced neurotoxicity. Slices following culturing for 13-15 days in culture media were treated with KA (300 μM, 1 hr) and incubated with lymphocytes beneath the insert membrane for 18 hours in the presence of PI (2 μM). (A) Representative PI fluorescence images showing cell death in slices treated with KA and incubated with lymphocyte preparations containing either non-activated or activated T cells for 18 hours. (B) Bar chart showing the reduction of cell death in slices co-cultured with lymphocyte preparations containing either non-activated T cells or activated T cells. All experiments were carried out in at least 15 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus KA (300 μM). Scale bar: 200 μm.

(A)



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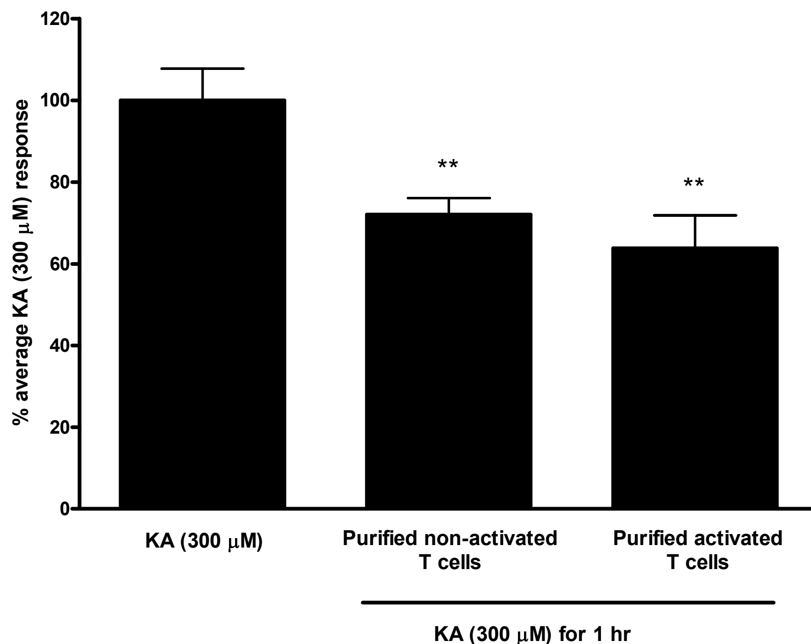


Figure 6.7 Neuroprotection of T cells against KA-induced cell death is independent of their activation state.

(A) Representative PI fluorescence images of slices treated with KA (300 μM) for 1 hour and co-cultured with either purified non-activated T cells or purified activated T cells for 18 hours. (B) Bar charts showing the neuroprotective response of purified non-activated T cells and purified activated T cells against KA 300 μM. All experiments were carried out in at least 6 separate animal preparations. Data presented as mean ± SEM. **$p < 0.01$ versus KA 300 μM. Scale bar: 200 μm.

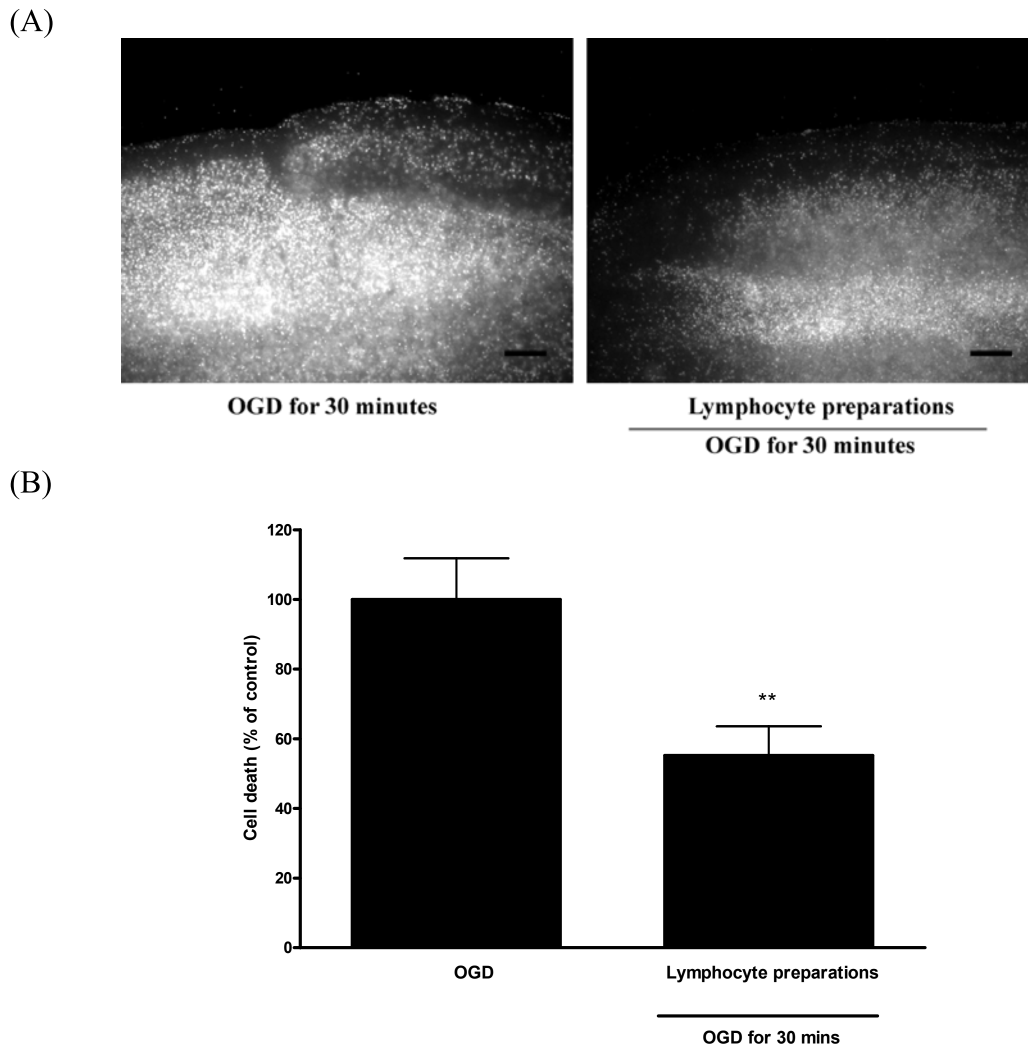


Figure 6.8 Lymphocytes exhibit neuroprotection against OGD-induced cell death. Slices after culturing for 13-15 days in prior to experiments, were subjected to OGD for 30 minutes and co-cultured with 1 X 10^6 lymphocytes for 18 hours in the presence of PI (2 μM). The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images of slices exposed to OGD for 30 minutes and incubated with either fresh culture media or media containing lymphocytes for 18 hours. (B) Bar chart showing that lymphocytes reduced the cell death induced by OGD. All experiments were carried out in at least 4 separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. **$p < 0.01$ versus 30 mins OGD. Scale bar: 200 μm.

6.2.4 Non-T cells also exhibit neuroprotection against KA-induced cell death

Having demonstrated a neuroprotective role of non-T cells against KA-induced cell death in hippocampus, I also investigated their role in the cortex of OSCs. Slices were treated with KA (300 μM, 1 hour) and incubated with either non-T cells or purified non-activated T cells for 18 hours. Similar to the hippocampus, non-T cells were also neuroprotective against KA-induced cell death (77.3 ± 5.0 % of the KA 300 μM control; n = 16 slices from 9 animals; $p < 0.05$; Figure 6.9) and also similar to the purified non-activated T cells (78.8 ± 4.2 % of the KA 300 μM control; n = 20 slices from 10 animals; $p < 0.05$) suggesting that non-T cells also contribute in the observed neuroprotection as seen in Figure 6.6. As a control experiment, slices were treated with KA for 1 hour and incubated with lymphocyte preparations for 18 hours. Consistent with the previous result (Figure 6.6), the lymphocyte preparations showed neuroprotection against KA-induced toxicity (42.6 ± 4.6 % of the KA 300 μM control; n =8 slices from 6 animals; $p < 0.001$).

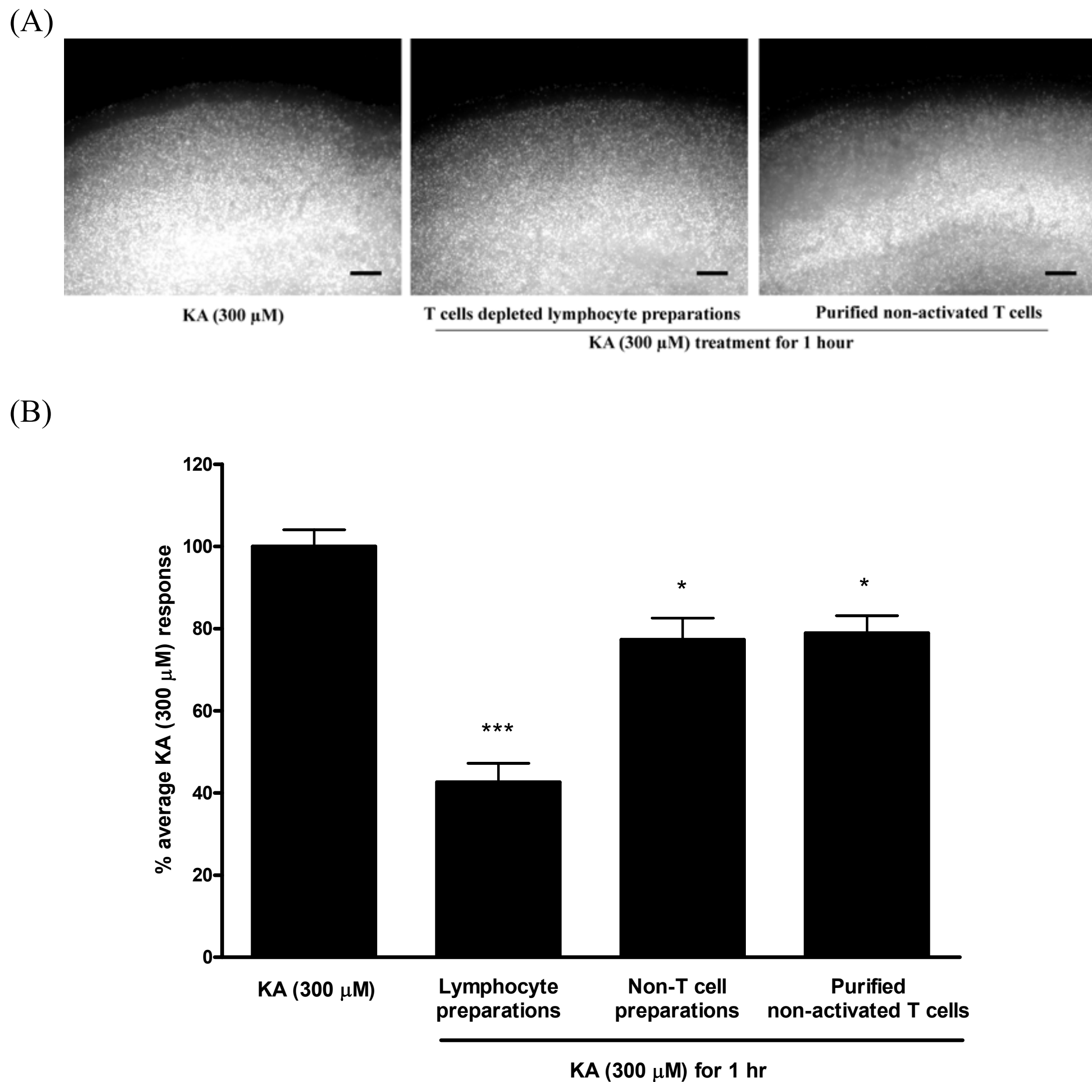


Figure 6.9 T cell depleted lymphocyte preparations (non-T cells) show a similar level of neuroprotection as purified non-activated T cells.

OSCs were treated as described in Figure 6.6. (A) Representative PI fluorescence images of slices treated with KA (300 μM) for 1 hour and co-cultured with either non-T cells or purified non-activated T cells for 18 hours. (B) Summary showing the response of lymphocyte preparations, non-T cells and purified non-activated T cells against KA-induced toxicity. All experiments were carried out in at least 6 different animal preparations. Data presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ versus KA 300 μM. Scale bar: 200 μm.

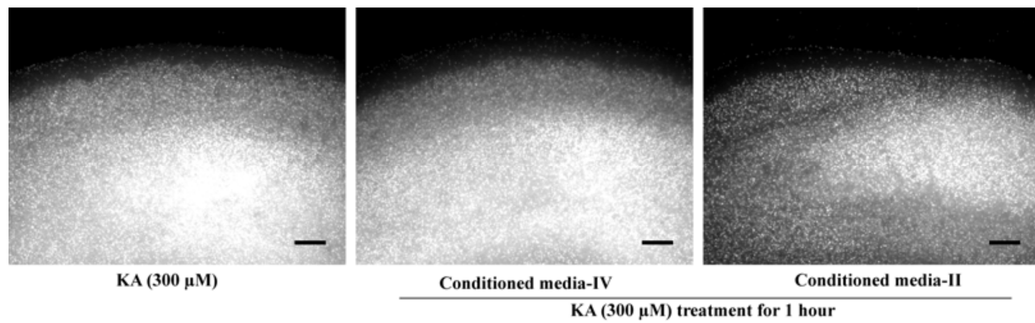
6.2.5 Soluble mediators secreted by lymphocytes are responsible for neuroprotection in KA-induced cell death.

Having established that conditioned media from lymphocyte preparations is neuroprotective in the hippocampus, I investigated the response of different types of conditioned media against KA-induced neurotoxicity in the cortex of OSCs. Slices following KA (300 μM, 1 hr) treatment were incubated in either conditioned media-I (conditioned media obtained from lymphocyte preparations exposed to OSCs) or conditioned media-II (conditioned media which were obtained from lymphocyte preparations without exposure to OSCs) with PI (2 μM) for 18 hours. Both conditioned media-I (69.5 ± 7.1 % of the KA 300 μM control; n =11 slices from 5 animals; $p < 0.001$; Figure 6.10) and conditioned media-II (60.8 ± 3.2 % of the KA 300 μM control; n = 10 slices from 5 animals; $p < 0.001$) showed neuroprotection against KA-induced cell death and no significant differences were found in the level of neuroprotection between conditioned media-I and conditioned media-II. Thus this suggests that conditioned media-II is equally effective as conditioned media-I. Hence, for the rest of these experiments, I used conditioned media-II. To determine whether the conditioned media alone from lymphocyte preparations are neuroprotective, I also investigated the response of conditioned media-III (conditioned media obtained from KA-treated slice) and conditioned media-IV (conditioned media obtained from rat primary hippocampal culture) against KA-induced cell death. OSCs were treated with KA and incubated in either conditioned media-III or conditioned media-IV for 18 hours. Neither conditioned media-III (97.6 ± 6.5 % of the KA 300 μM control; n = 9 slices from 6 animals; $p > 0.05$ versus KA 300 μM) nor conditioned media-IV (101.8 ± 4.4 % of the KA 300 μM control; n = 9 slices from 5 animals; $p > 0.05$ versus KA 300 μM) showed neuroprotection against KA-induced cell death. These findings suggest that only those conditioned media from lymphocyte preparations are neuroprotective.

To investigate whether soluble mediators present in conditioned media-II are proteinaceous, conditioned media-II was heated which denatured the proteins present in

the media as described previously (see section 2.2.6.1.5). When OSCs were treated with KA and incubated in heated conditioned media-II, the observed neuroprotection was abolished (89.5 ± 5.2 % of the KA 300 μM control; n = 6 slices from 5 animals; $p > 0.05$ versus KA 300 μM; Figure 6.11) suggesting that lymphocytes release soluble mediators are proteins and are likely to be responsible for the neuroprotection.

(A)



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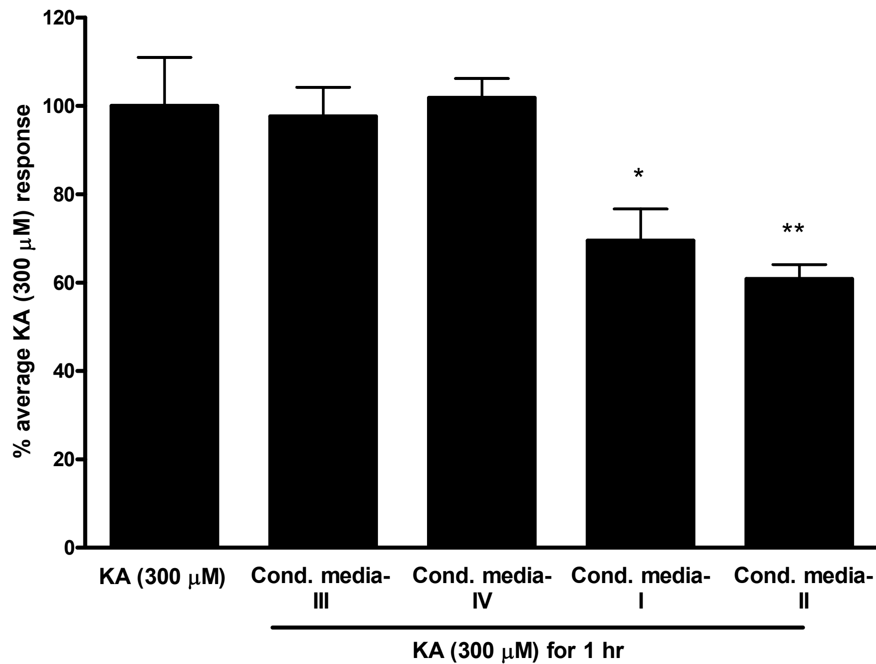


Figure 6.10 Soluble mediators released by lymphocytes are neuroprotective.

OSCs were treated as described in Figure 6.6. (A) Representative PI fluorescence images of slices treated with KA (300 µM) for 1 hour and incubated in either conditioned media-I or conditioned media-II or conditioned media-IV for 18 hours. (B) Bar chart demonstrating the neuroprotection exhibited by conditioned media-I and II but not by conditioned media-III and IV. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$ versus KA 300 µM. Scale bar: 200 µm.

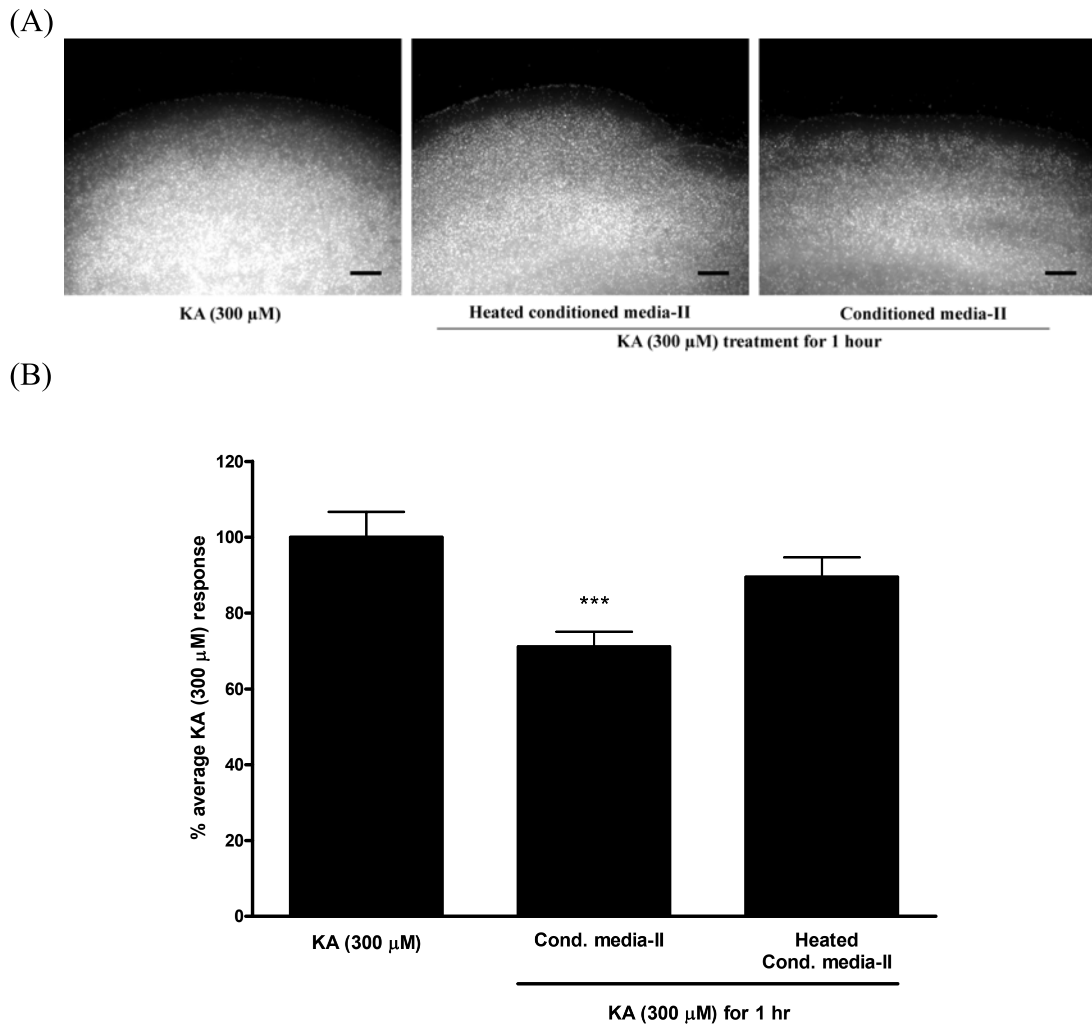


Figure 6.11 Heated conditioned media is not neuroprotective against KA-induced toxicity.

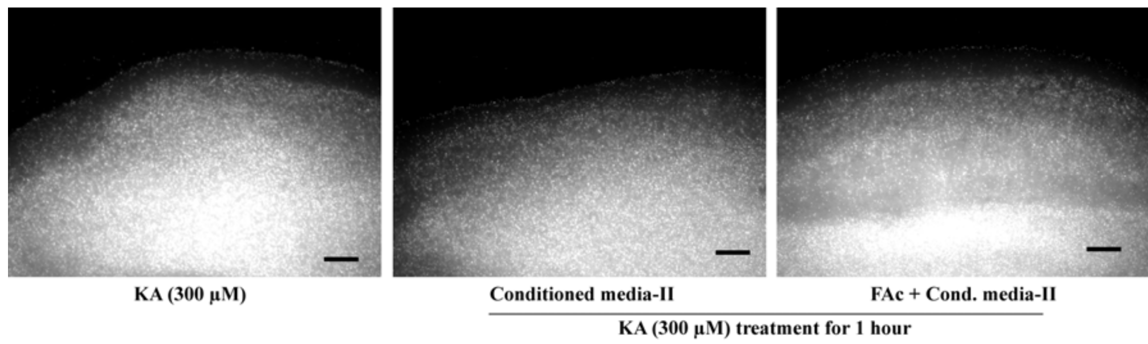
Slices were treated as previously described (Figure 6.6). (A) Representative PI fluorescence images of slices pre-treated with KA (300 μM) for 1 hour and incubated in either conditioned media-II or heated conditioned media-II for 18 hours. (B) Bar chart demonstrating that heated conditioned media-II failed to exhibit neuroprotection against KA-induced neurotoxicity whereas conditioned media-II showed neuroprotection against KA-induced toxicity. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. ***$p < 0.001$ versus KA 300 μM. Scale bar: 200 μm.

6.2.6 Inhibition of astrocytic function blocks lymphocyte-mediated neuroprotection in KA-induced cell death and in OGD-induced cell death.

Having demonstrated the neuroprotective effect of the conditioned media obtained from lymphocytes, I investigated the involvement of astrocytes in cortex of OSCs by selectively inhibiting the astrocytic function using FAc, the glial cell metabolic inhibitor (Swanson and Graham, 1994; Fonnum *et al.*, 1997). OSCs were pre-treated with a glial cell metabolic inhibitor (FAc, 10 µM) for 2 hours which were subsequently treated with KA (300µM, 1 hr) in the presence of FAc and incubated in conditioned media-II (conditioned media obtained from lymphocytes without exposure to OSCs) in the presence of FAc for 18 hours. The conditioned media in the presence of FAc was not neuroprotective (94.1 ± 2.2 % of the KA 300 µM control; n = 21 slices from 10 animals; Figure 6.12) whereas conditioned media-II alone significantly reduced KA-induced cell death (78.8 ± 4.1 % of the KA 300 µM control; n = 27 slices from 12;animals $p < 0.01$).

However, the present study showed a contrasting result with regards to OGD-induced cell death. Slices were pre-treated with FAc for 2 hours 30 minutes which were subsequently subjected to OGD for 30 minutes in the presence of FAc and incubated in conditioned media-II with FAc for 18 hours. The effect of conditioned media-II was reversed in conditioned media-II with FAc (90.8 ± 6.7 % of 30 mins OGD control; n = 5 slices from 3 animals; Figure 6.13) when compared with conditioned media-II alone (76.3 ± 1.0 % of 30 mins OGD control; n = 4 slices from 3 animals).

(A)



(B)

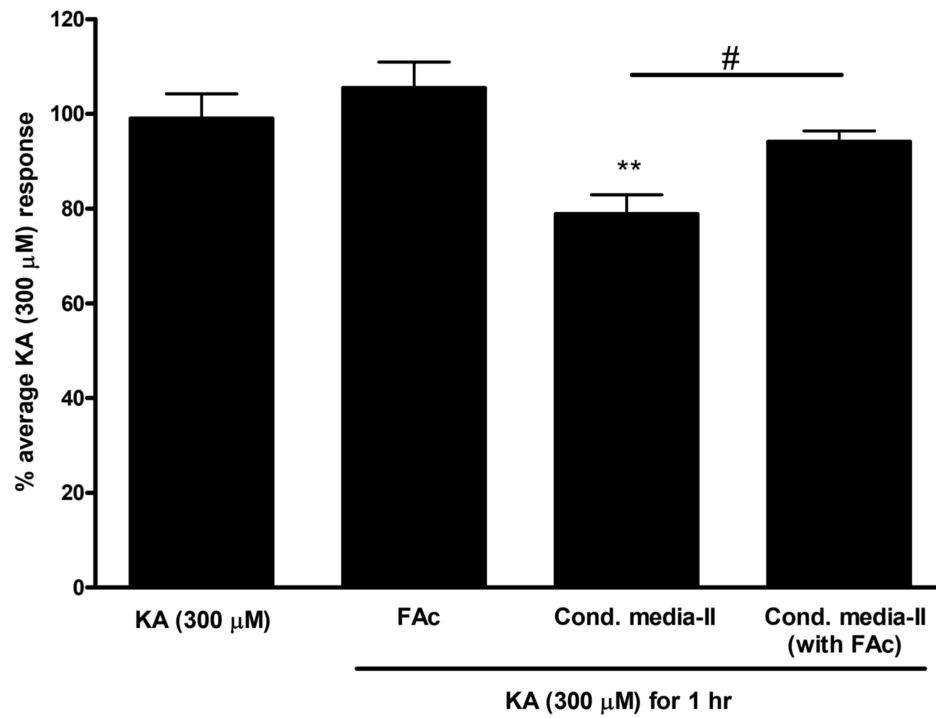


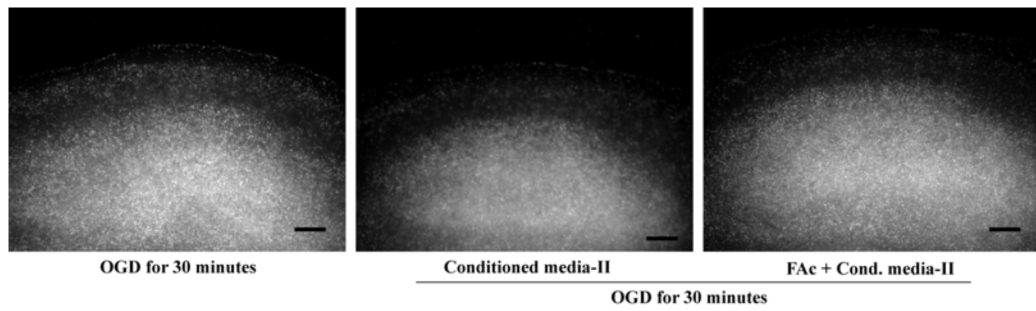
Figure 6.12 The neuroprotection mediated by conditioned media from lymphocytes is abolished after the inhibition of astrocytic function.

Continued…

Figure 6.12 The neuroprotection mediated by conditioned media from lymphocytes is abolished after the inhibition of astrocytic function.

Slices following cultured for 13-15 days were pre-treated with FAc for 2 hours and subsequently treated for 1 hour with KA in the presence of FAc which were then incubated in conditioned media-II for 18 hours either in the presence or absence of FAc with PI. (A) Representative PI fluorescence images showing cell death in slices treated with KA following either FAc pre-treatment or without treatment which were then incubated in conditioned media-II either in the presence or absence of FAc for 18 hours. Control slices were treated with KA (300 μM, 1 hr) in the absence of FAc and incubated in fresh culture media for 18 hours (B) Bar chart illustrating the reversal of neuroprotection after inhibition of astrocytic function. All experiments were carried out in at least 10 separate animal preparations. Data presented as mean ± SEM. #$p < 0.05$ versus FAc treated slice, **$p < 0.01$ versus 300 μM KA. Scale bar: 200 μm.

(A)



(B)

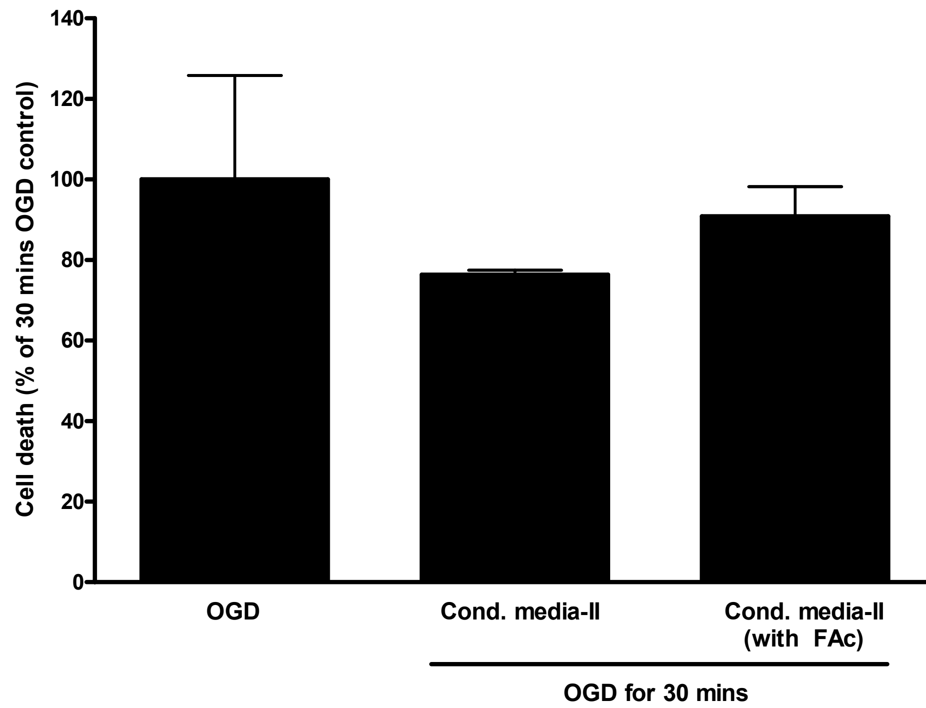


Figure 6.13 Inhibition of astrocytic function reverses the effect of conditioned media.

Continued…

Figure 6.13 Inhibition of astrocytic function reverses the effect of conditioned media.

Slices were cultured for 13-15 days prior to experiments. Slices after FAc pre-treatment (2 hours 30 minutes) were subjected to OGD for 30 minutes in the presence of FAc (10 μM) for 30 minutes and incubated in conditioned media-II with PI for 18 hours in the presence of FAc. The PI images were visualised by fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in FAc pre-treated slices which were incubated in conditioned media-II either in the presence or absence of FAc for 18 hours. Control slices were treated in OGD condition for 30 minutes and incubated in fresh culture media for 18 hours. (B) Bar chart showing significantly more cell death in the conditioned media with FAc when compared to the conditioned media alone. All experiments were carried out in at least 3 separate animal preparations. Data presented as mean ± SEM. Scale bar: 200 μm.

6.2.7 Inhibition of ERK and p38 MAP kinase pathways underlie the lymphocyte-mediated neuroprotection

In the previous chapter, I showed that ERK and p38 MAP kinase activity was reduced in OSCs when co-cultured with lymphocytes by western blotting. Here, I have investigated whether inhibition of these MAP kinases can modulate the effect of either KA-induced cell death or OGD-induced cell death by a pharmacological approach. I first investigated KA-induced cell death in the presence of U0126 (20 μM), an ERK inhibitor and SB203580 (100 μM), a p38 MAP kinase inhibitor to mimic lymphocytes-induced reduction in MAP kinase activity. Slices were treated with KA (300 μM, 1 hr) and incubated in fresh media with PI (2 μM) containing U0126 and SB23580 for 18 hours. Both MAP kinase inhibitors showed significant decreases in KA-induced cell death (84.3 ± 5.0 % of the KA 300 μM + vehicle control; $n = 11$ slices from 6 animals; $p < 0.05$; Figure 6.14).

Similarly, when slices were subjected to OGD conditions for 30 minutes and incubated in the fresh media containing MAP kinase inhibitors (U0126 and SB203580), MAP kinase inhibitors neuroprotective against OGD-induced cell death (63.3 ± 10.6 % of 30 mins OGD + vehicle control; $n = 9$ slices from 4 animals; $p < 0.05$; Figure 6.15). This suggests that in the KA model and the OGD model, lymphocyte-mediated neuroprotection at least partially underlie the inhibition of MAP kinase activity.

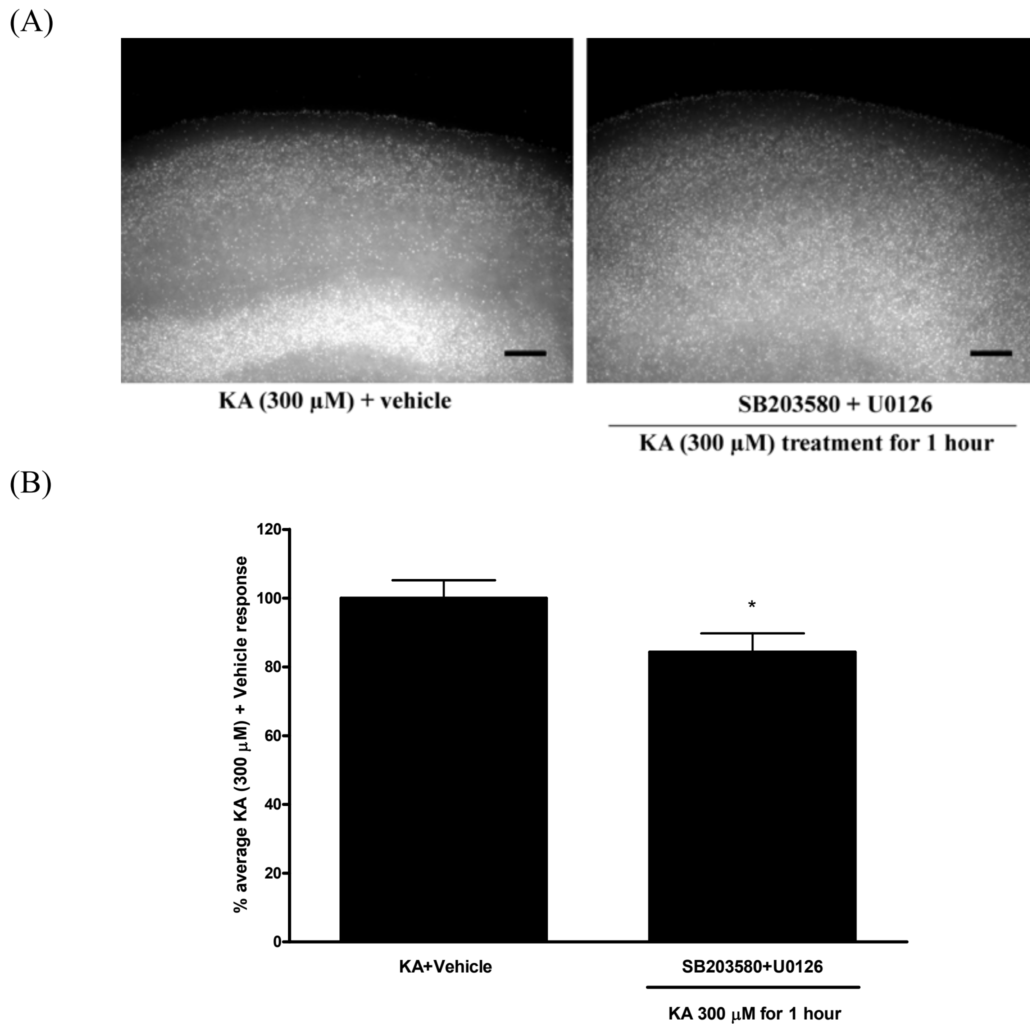


Figure 6.14 Pharmacological inhibition of MAP kinases inhibit KA-induced toxicity.

Slices were cultured for 13-15 days prior to experiments and treated with KA 300 µM for 1 hour and incubated in fresh culture media with MAP kinase inhibitors for 18 hours. Here MAP kinase inhibitors were used to mimic lymphocytes-induced reduction in MAP kinase activity. (A) Representative PI fluorescence images showing cell death in slices treated with KA and incubated in the fresh culture media either in the presence or absence of MAP kinase inhibitors for 18 hours. (B) Bar chart demonstrating the reduction in cell death by SB203580 and U0126. All experiments were carried out in 6 separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. $*p < 0.05$ versus 300 µM KA + vehicle. Scale bar: 200 µm.

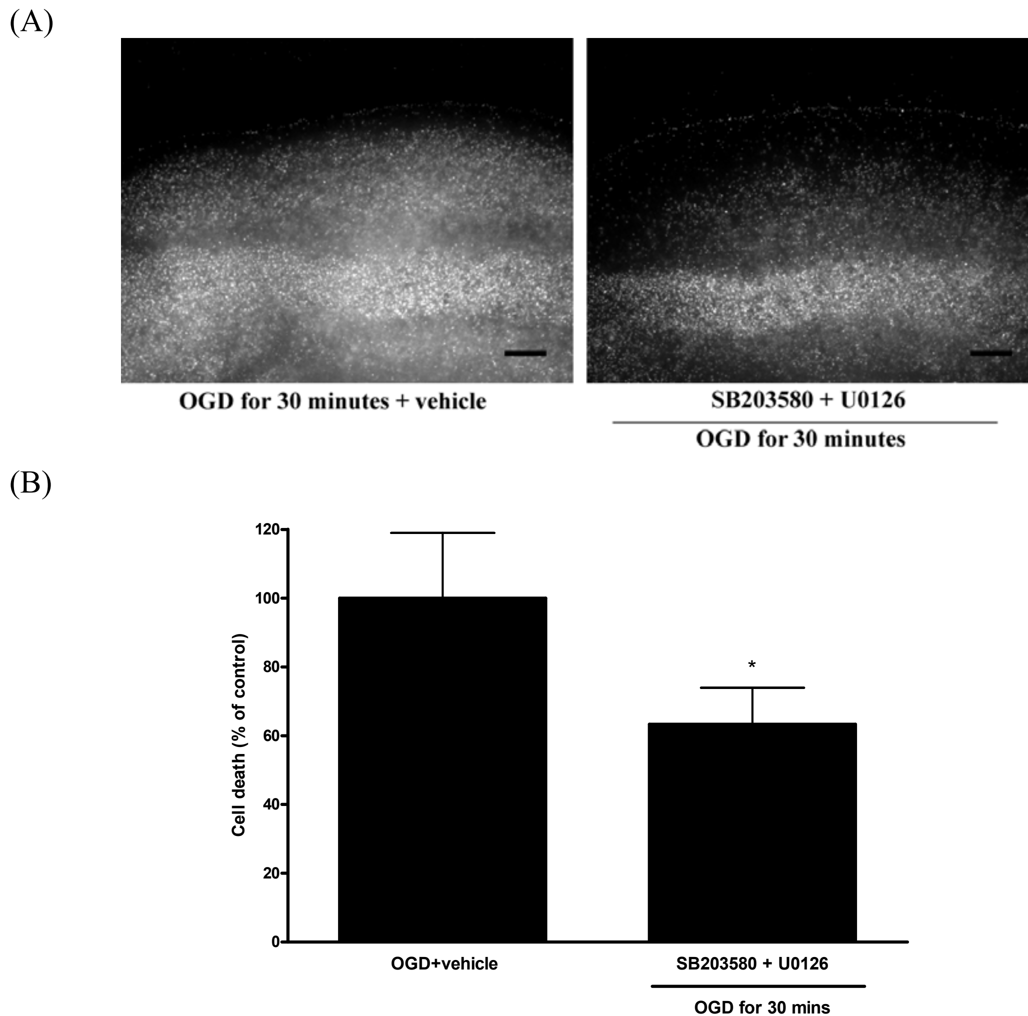


Figure 6.15 Pharmacological inhibition of MAP kinases inhibit OGD-induced toxicity.

Slices were subjected to OGD for 30 minutes and incubated in fresh culture media with MAP kinase inhibitors for 18 hours. MAP kinase inhibitors were used to mimic lymphocyte-induced reduction in MAP kinase activity. (A) Representative PI fluorescence images showing cell death in OSCS exposed to OGD for 30 minutes and incubated in fresh culture media either in the presence or absence of MAP kinase inhibitors for 18 hours. (B) Summary illustrating SB203580 and U0126 reducing cell death induced by OGD. All experiments were carried out in at least 4 separate animal preparations. Data were analysed using unpaired t-test and presented as mean ± SEM. *$p < 0.05$ versus OGD 30 minutes + vehicle. Scale bar: 200 μm.

6.2.8 The PI-3 kinase signalling pathway is not involved in the observed neuroprotection in KA-induced cell death.

Having established that MAP kinase signalling pathways are involved in the lymphocyte-mediated neuroprotection, I also investigated PI-3 kinase signalling pathway using PI-3 kinase inhibitor (LY294002, 10 μM) to determine whether this signalling pathway is involved in the observed neuroprotection. Slices were treated with KA (300 μM, 1 hr) in the presence of the PI-3 kinase inhibitor and incubated in conditioned media-II with PI-3 kinase inhibitor for 18 hours. The conditioned media in the presence of PI-3 kinase inhibitor showed neuroprotection against KA-induced neurotoxicity (83.8 ± 4.2 % of the KA 300 μM control; n = 6 slices from 6 animals; $p < 0.05$) which was similar to the neuroprotection exhibited by the conditioned media alone (74.0 ± 5.1 % of the KA 300 μM control; n =13 slices from 6 animals; $p < 0.05$; Figure 6.15). This suggests that PI-3 kinase signalling pathway does not underlie the neuroprotection mediated by the conditioned media from lymphocytes.

Next I also investigated the role of PI-3 kinase in the OGD model. Slices were exposed to OGD for 30 minutes in the presence of PI-3 kinase inhibitor and transferred to conditioned media-II containing PI-3 kinase inhibitor for 18 hours. Similar to KA-induced cell death, the conditioned media in the presence of PI-3 kinase inhibitor showed neuroprotection against OGD-induced cell death (63.1 ± 5.9 % of 30 mins OGD control; n = 6 slices from 4 animals; $p < 0.05$) which is similar to the conditioned media alone (76.3 ± 1.0 % of 30 mins OGD control; n = 4 slices from 3 animals; $p < 0.05$).

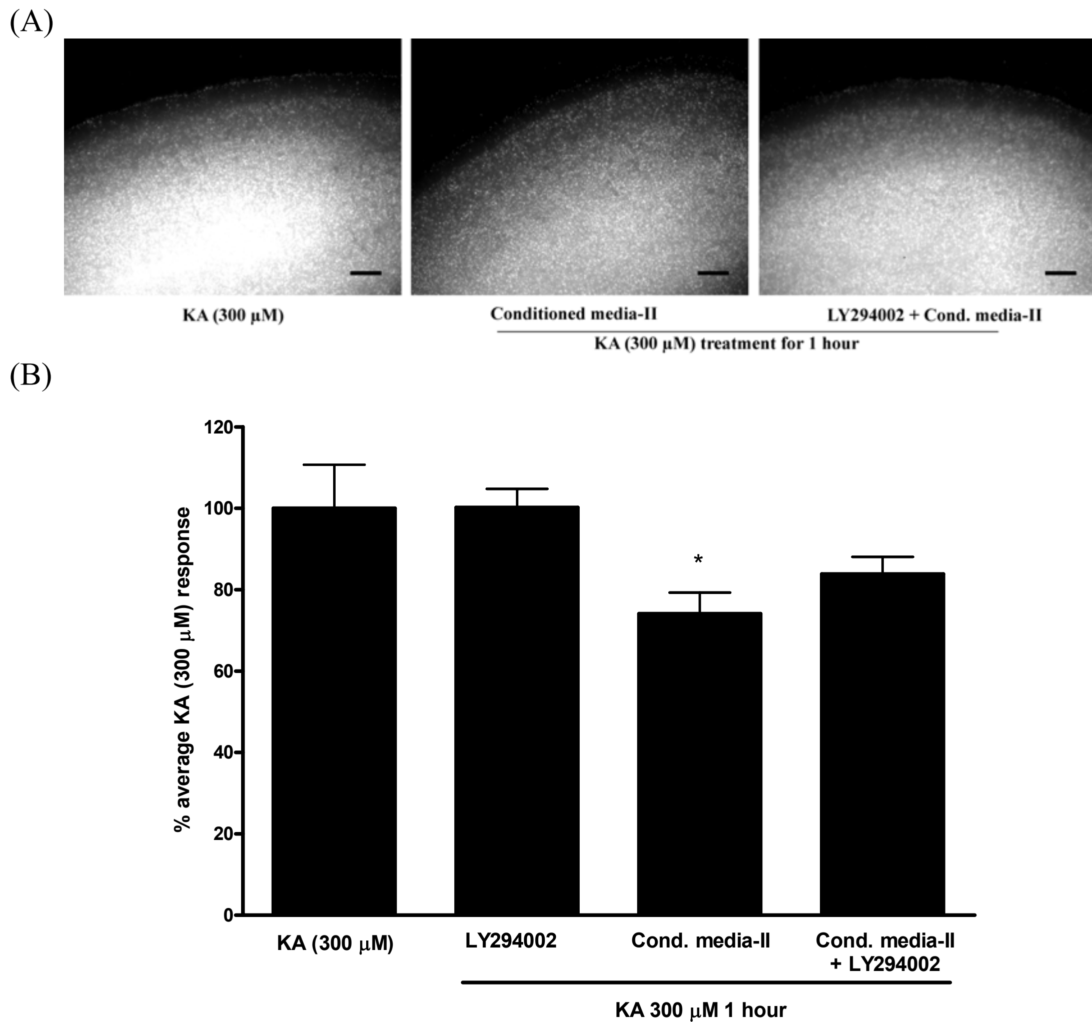


Figure 6.16 The neuroprotection mediated by the conditioned media from lymphocytes does not involve the PI-3 kinase signalling pathway in the KA model. Slices which were cultured for 13-15 days prior to experiments were treated with KA and LY294002 for 1 hour and incubated in conditioned media-II with LY294002 for 18 hours. The PI images were visualised using fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in slices treated with KA (300 μM, 1 hr) alone, conditioned media in the presence of PI-3 kinase and conditioned media alone. (B) Bar chart showing the reduction in cell death either in the presence or absence of PI-3 kinase inhibitor. All experiments were carried out in at least 5 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$ versus KA 300 μM. Scale bar: 200 μm.

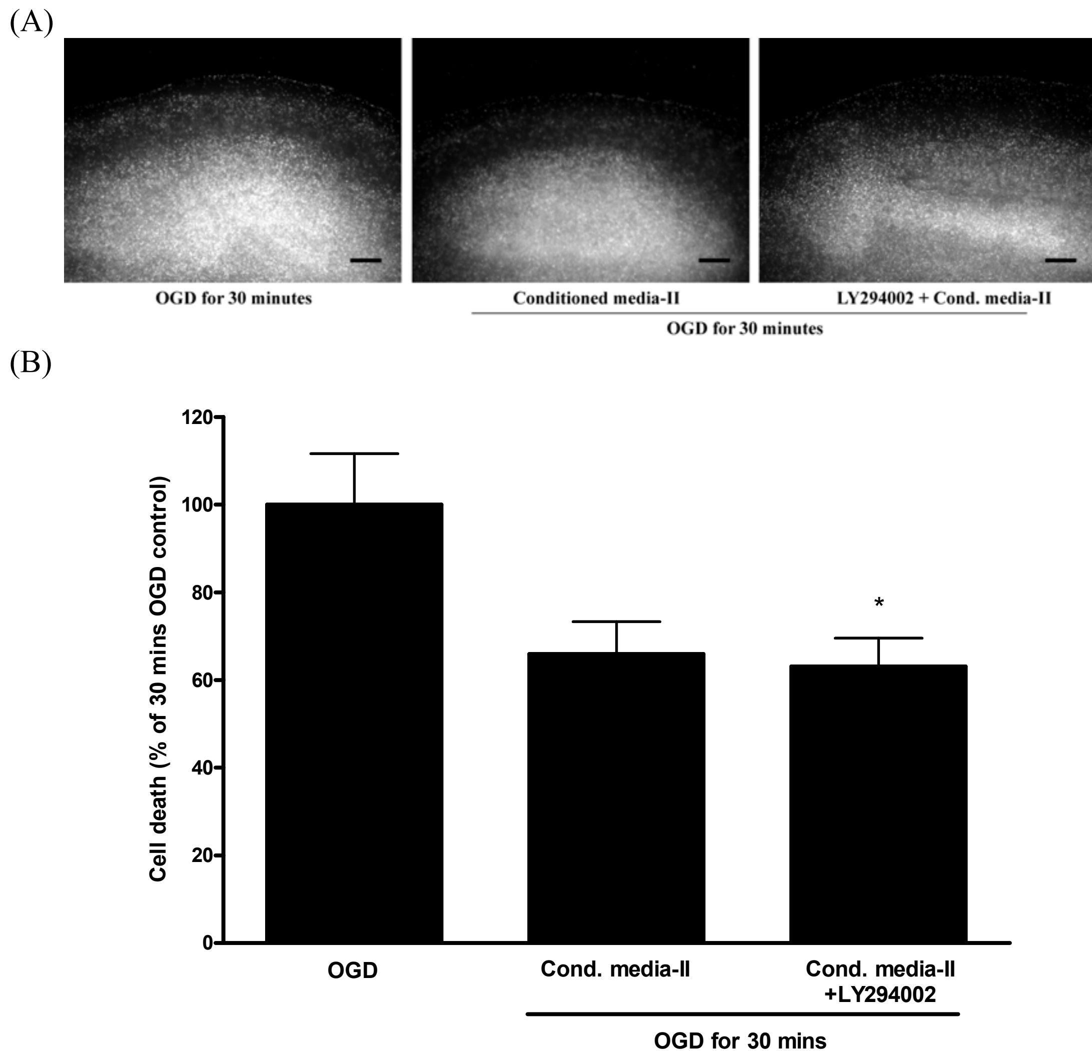


Figure 6.17 PI-3 kinase signalling pathway is not involved in the neuroprotection mediated by the conditioned media from lymphocytes in OGD-induced cell death. Slices were exposed to OGD for 30 minutes in the presence of LY294002 and incubated in conditioned media with LY294002 for 18 hours. The PI images were visualised using fluorescence microscopy. (A) Representative PI fluorescence images showing cell death in OSCs exposed to OGD, conditioned media with LY294002 and conditioned media alone. (B) Summary illustrating the effect of conditioned media either in the presence or absence of PI-3 kinase inhibitor. All experiments were carried out in at least 3 separate animal preparations. Data presented as mean ± SEM. *$p < 0.05$ versus OGD 30 minutes. Scale bar: 200 μm.

6.3 Discussion

Here in this chapter, I investigated the role of lymphocytes in the cortex of OSCs and sought for any difference between the cortex of OSCs and the hippocampus. Consistent with the hippocampus data, lymphocytes show neuroprotection against KA-induced cell death and OGD-induced cell death in the cortex of OSCs and the lymphocyte-mediated neuroprotection is independent of activation state of T cells since both non-activated T cell and activated T cells within lymphocyte preparations show a similar level of neuroprotection. The present data also showed that the observed neuroprotection is contact-independent since the conditioned media from lymphocytes are neuroprotective which suggests that soluble mediators released by lymphocytes are responsible for the observed neuroprotection. Moreover, I also showed that soluble mediators are proteinaceous as heated conditioned media loses its neuroprotective property. For detail discussion, please refer to chapter 4 (section 4.3).

Having established that lymphocytes are neuroprotective, I also investigated the role of astrocytes as astrocytes play a key role during neuroinflammation (Brambilla *et al.*, 2005; van Loo *et al.*, 2006). Here, I showed that the inhibition of astrocytic function using FAc, a glial cell metabolic inhibitor has abolished the lymphocyte-mediated neuroprotection in KA-induced cell death as well as in OGD-induced cell death which suggests that the astrocytic function has critical role in the observed neuroprotection. For further details, please refer to chapter 5 (section 5.3). However, it is also noteworthy that contrary to the hippocampus, the inhibition of astrocytic function reversed the effect of the conditioned media in the OGD model. Thus suggests that astrocytes in the cortex of OSCs might have potential role in the neuroprotection mediated by conditioned media from lymphocytes. A possible explanation of this inconsistent finding might be due to the difference in the sensitivity of astrocytes to OGD in the cortex of OSCs and the hippocampus. However, in reviewing the literature, no data was found on comparison of sensitivity of these cells according to their location. Therefore, further work is required to establish this.

As described earlier (see chapter 5), the reliable evidence for the underlying cellular signalling mechanism(s) of the lymphocyte-mediated neuroprotection is still poorly understood. Investigation of MAP kinase signalling pathways by western blot showed the reduction of ERK and p38 activities which was also demonstrated using their specific inhibitors to mimic lymphocytes-induced reduction in MAP kinase activity which show the neuroprotection against KA-induced neurotoxicity as well as against OGD induced cell death. These findings are consistent with hippocampal results which suggest that lymphocyte-mediated neuroprotection involved the inhibition of ERK and p38 MAP kinase activity. For further discussion please refer to chapter 5 (section 5.3).

The present study has also investigated the PI-3 kinase signalling pathway. Consistent with hippocampal results, PI-3 kinase do not have any role in neuroprotection mediated by conditioned media from lymphocytes in KA-induced cell death as well as in OGD-induced cell death since PI-3 kinase inhibitor fails to block the observed neuroprotection. Please refer to chapter 5 (section 5.3) for the discussion.

In summary, a similar neuroprotective response of lymphocytes against KA-induced cell death and OGD-induced cell death has been demonstrated in the cortex of OSCs as of the hippocampus. However, the inhibition of astrocytes has reversed the effect of the conditioned media from lymphocytes in the OGD model which is contrast to the hippocampus. Nevertheless, to the best of my knowledge, this is the first time the current study has highlighted the involvement of MAP kinase signalling pathways in the lymphocyte-mediated neuroprotection. This suggests that lymphocyte-mediated neuroprotection underlie the inhibition of ERK and p38 MAP kinase activity.

7 General Discussion

The overall aim of the present study was to investigate the role of lymphocytes in neurodegenerative diseases and determine the underlying mechanism(s) of that response. Hence, in an attempt to address these issues, I have investigated the role of lymphocytes in two neurodegenerative disease models (the KA model and the OGD model) using organotypic cortico-hippocampal slice cultures and examined whether there is any difference in the response of lymphocytes in these two models. However, it was also important to develop a viable experimental setup to test the hypothesis (see Chapter 3). The present study therefore first examined the response of slices to lymphocytes under normal culture conditions before inducing cell death to determine whether lymphocytes induce any toxicity to slices. In addition, I also compared the role of lymphocytes in the hippocampus and the cortex of OSCs as slice cultures contain intact hippocampus and cortex (see Figure 6.1).

My first aim was to examine the role of lymphocytes in normal and diseased state in the CNS. Therefore, I used normal culture conditions to address the state of the CNS while the KA model and the OGD model were used to address diseased states. The present data has revealed that under normal culture conditions, lymphocytes do not induce toxicity to the hippocampus and the cortex of OSCs. In support of this finding, Wolf *et al.* (2002) have also demonstrated that T cells when added beneath slices separated by the insert membrane were not toxic. I have also shown that the presence of either non-activated or activated T cells within lymphocyte preparations do not have any effect in the response of lymphocytes to OSC under normal culture condition. Consistent with this finding, several *in vivo* studies have shown that peripherally activated lymphocytes enter the healthy CNS via the BBB (Hickey, 1991; Hickey *et al.*, 1991; Engelhardt and Ransohoff, 2005) but do not initiate any inflammatory response untill they recognise their cognate antigens and those lymphocytes which are unable to recognise the antigens exit from the CNS regularly (Hickey *et al.*, 1991; Becher *et al.*, 2006; Odoardi *et al.*, 2007; Bartholomaus *et al.*, 2009). Further, there is also evidence for infiltration of non-

activated lymphocytes into the CNS via the blood-CSF barrier and are suggested to be a normal recirculating lymphocyte pool of the CSF (Seabrook *et al.*, 1998). When taken together these findings suggested that in the normal CNS, lymphocyte preparations containing either non-activated or activated T cells do not initiate inflammatory responses.

Having established that lymphocytes do not induce toxicity to OSCs under the normal culture condition, continuing to address my first aim, I investigated the role of lymphocytes in neurodegenerative diseases using two disease models. I showed that lymphocyte preparations are neuroprotective against both KA-induced toxicity and OGD-induced cell death in the hippocampus and the cortex of OSCs. Although, various studies have demonstrated the neuroprotective role of T cells (Schwartz, 2005; Beers *et al.*, 2008) and B cells (Fillatreau *et al.*, 2002; Kala *et al.*, 2010) in neurodegenerative diseases, no literature has been found discussing the role of lymphocytes preparations. However, in a recent *in vivo* study in RAG1 knock-out mice (T and B cells deficient mice) who showed more neurodegeneration with spontaneous recurrent seizures following KA injection in comparison to saline injected RAG1 knock-out and wild type controls (Zattoni *et al.*, 2011). Zattoni *et al.* (2011) have suggested that adaptive immune response can modulate neuronal damage and spontaneous recurrent seizures in KA-lesioned hippocampus. Therefore, a possible explanation for the neuroprotective response of lymphocyte preparations might be due to the presence of T cells, B cells and other immune cells including macrophages and natural killer cells as a neuroprotective role of these cells have been demonstrated by several studies (Moalem *et al.*, 1999; Hammarberg *et al.*, 2000; Wolf *et al.*, 2002; Beers *et al.*, 2008). Supporting this argument, flow cytometry revealed that the majority of cells in lymphocyte preparations were found to be T cells and after purifying T cells from lymphocyte preparations, I was able to show that both non-activated and purified activated purified T cells are neuroprotective without having any significant differences in the level of neuroprotection. This suggests that T cells are neuroprotective but the observed neuroprotection is independent of the activation state of T cells. Although, the

infiltration of non-activated T cells into the CNS has been shown (Seabrook *et al.*, 1998), previous reports have not investigated their role in either normal or disease state of the CNS. Thus, the present study has highlighted a potentially novel role for non-activated T cells during neurodegeneration. However, these data must be interpreted with caution because the role of non-activated T cells in *in vitro* and *in vivo* disease models might be different and further investigation is necessary to determine their contribution in *in vivo* diseased state. The current study has also demonstrated the neuroprotective role of purified activated T cells which corroborate the findings of a great deal of the previous work in this field (Moalem *et al.*, 1999; Wolf *et al.*, 2002; Schwartz, 2005; Beers *et al.*, 2008). The adoptive transfer of auto-reactive T cells from mice immunised with myelin-basic protein (a self-antigen which induces EAE) to mice with partial optic nerve crush (a model for secondary neurodegeneration) showed more optic nerves survival which resisted to secondary neurodegeneration (Moalem *et al.*, 1999). Similar observations have been shown that the administration of auto-reactive Th-1 cells to EAE-induced or optical nerve crush mice exhibited more neuronal survival in compare to PBS injected controls (Kipnis *et al.*, 2002). The neuroprotective role of T cells has also been demonstrated in T cell and B cell deficient mice, SCID mice (Serpe *et al.*, 1999, 2003). After facial nerve transaction, SCID mice manifested severe impairment of facial motor neurons which were restored up to control levels following adoptive transfer of wild-type splenocytes containing T and B cells (Serpe *et al.*, 1999, 2003) suggesting potential role of lymphocytes in protecting from secondary neurodegeneration. In addition, supporting the present data, *in vitro* study in murine entorhinal-hippocampal brain slices has shown better survival of neurons when incubated with Th-1 and Th-2 cells (Wolf *et al.*, 2002). However, this study didn't induce neurodegeneration. Moreover, differentiation of activated T cells into T_{reg} cell and contributing to the observed neuroprotection under the present experimental conditions cannot be excluded as it has been illustrated that upon activation by their cognate antigen/MHC-II, $CD4^+$ T cells in the presence of TGF-β and IL-10 or IL-2 are able to differentiate into T_{reg} cells (Nakamura *et al.*, 2001; Chen *et al.*, 2003; Bettelli *et al.*, 2006). Hence, another possible explanation for the observed neuroprotection might

be due to the presence of T_{reg} cells which are suggested to have an immunosuppressive role during neuroinflammation (Liu *et al.*, 2006; Liu *et al.*, 2009; Reynolds *et al.*, 2010). However, the present study has not examined the presence of T_{reg} cells in lymphocyte preparations and a further study could assess their presence in lymphocyte preparations.

I further demonstrated that after T cells purification, remaining cells 'non-T cells' are also neuroprotective against KA-induced cell death in the hippocampus as well as in the cortex of OSCs. The flow cytometry showed that the majority of cells were B cells within non-T cell preparations. This suggests that neuroprotection elicited by non-T cells might be due to the presence of B cells. The present findings seem to be consistent with other research which has demonstrated the possible role of B cells in neurodegenerative diseases (Fillatreau *et al.*, 2002; Kala *et al.*, 2010; Berer *et al.*, 2011). The neuroprotective role of B cells has been suggested to be mediated via IL-10 production (Fillatreau *et al.*, 2002; Matsushita *et al.*, 2008; Carter *et al.*, 2011) since, mice lacking IL-10 producing B cells showed a diseases progression as well as an increase in number of pathogenic T cells and a decreased number of T_{reg} cells (Carter *et al.*, 2011). In addition, B cells have been shown to release various growth factors including BDNF, NT-3 and TGF-β and anti-inflammatory cytokines such as IL-4 and IL-10 (Kerschensteiner *et al.*, 1999; Edling *et al.*, 2004; Kala *et al.*, 2010) which can contribute to neuroprotection. Meanwhile, the contribution of other immune cells including macrophages and natural killer cells in neuroprotection cannot be excluded as various studies have shown their neuroprotective role in neurodegenerative diseases (Zhang *et al.*, 1997; Hammarberg *et al.*, 2000; Schwartz and Yoles, 2006; London *et al.*, 2011). A further study with more focus on these cells is therefore suggested.

The present study demonstrated that the lymphocyte-mediated neuroprotection is contact-independent since conditioned media from lymphocytes was neuroprotective. Supporting this finding, Wolf *et al.* (2002) have demonstrated that direct contact of T cells with OSCs was neurotoxic while OSCs co-cultured with T cells separated by an insert membrane were neuroprotective and hypothesised a contact-independent response

for T cells. The current study has further confirmed that soluble mediators present in the conditioned media are protein in nature as heating the conditioned media abrogates the neuroprotection. It seems possible that the observed neuroprotection exhibited by soluble mediators might be due to the presence of various neurotrophic factors, growth factors and anti-inflammatory mediators. Several studies have reported that lymphocytes release neurotrophic factors such as BDNF, NT-3 and NT-4/5 (Kerschensteiner *et al.*, 1999; Schuhmann *et al.*, 2005; Heese *et al.*, 2006) and these factors contribute to neuroprotection and repair of the injured nervous system (Sendtner *et al.*, 1992; Yan *et al.*, 1992; McTigue *et al.*, 1998; Culmsee *et al.*, 2002). In addition to neurotrophic factors, lymphocytes also release neuroprotective cytokines including IL-4 and IL-10 and growth factors like TGF and insulin-like growth factor (IGF) (Zhu *et al.*, 2002; Zhao *et al.*, 2006; Schwartz and Ziv, 2008; Ouyang *et al.*, 2011; Xin *et al.*, 2011) which may underlie the neuroprotection contributed by the conditioned media. However, the identification of the exact mediator(s) was not examined in the present study.

As mentioned previously, one of the important aims of this research is to examine whether lymphocytes play different role depending on the disease model. I therefore, investigated the role of lymphocytes in the OGD model and have shown neuroprotection similar to the KA model. Although, various *in vivo* studies (Hurn *et al.*, 2007; Liesz *et al.*, 2011) have reported the neurotoxic effect of lymphocytes in stroke, the present data has shown that lymphocytes are neuroprotective in OGD under the present experimental conditions. To the best of my knowledge, I demonstrated for the first time the neuroprotective role of lymphocytes in an *in vitro* model of stroke which is contact-independent. Supporting these findings, several *in vivo* studies have demonstrated the neuroprotective role of lymphocytes in stroke (Frenkel *et al.*, 2005; Planas and Chamorro, 2009; Ren *et al.*, 2011). The infarct size of the brain is found to be significantly reduced in the mice with adoptive transfer of MOG-specific $CD4^+$ T cells (Frenkel *et al.*, 2005). A possible explanation for the neuroprotective role of lymphocytes under the present study might be due to the presence of IL-10 and TGF-β released by T cells and B cells. IL-10 has also been suggested to be a modulator of

activated macrophage/microglia and astrocytes thus limiting secondary inflammatory processes (Arumugam *et al.*, 2005) and also reported to be protective against glutamate toxicity (Bachis *et al.*, 2001). IL-10 secreted by $CD4^{+}$ T cells has been suggested to be responsible for reduction in the infarct size of the brain during stroke (Frenkel *et al.*, 2003; Frenkel *et al.*, 2005). In addition, B cells have also been found to be the major IL-10 producing cells which help in reducing CNS inflammation and neurological deficit during stroke (Ren *et al.*, 2011) since the adoptive transfer of B cells from wild-type to B cell deficient mice prevented ischemia while transfer of B cells from IL-10 $^{-/-}$ mice to B cell deficient mice fails to prevent it. The low level of IL-10 in elderly patients with a history of stroke in comparison to elderly people without stroke further supports the potential role of IL-10 during stroke (van Exel *et al.*, 2002; Vila *et al.*, 2003) as lower plasma concentrations of IL-10 were correlated with patients having worst neurological symptoms following stroke (Vila *et al.*, 2003). Recently, the role of T_{reg} cells has been highlighted in stroke. T_{reg} cells have been suggested to be neuroprotective in stroke (Liesz *et al.*, 2009; Planas and Chamorro 2009) and are reported to be involved in reducing the production of pro-inflammatory cytokines including TNF-α, IFN-γ and IL-1β, inhibition of neutrophils infiltration into the brain and modulate the activation of microglia in the ischemic brain (Liesz *et al.*, 2009). Leisz *et al.* (2009) further suggested that the neuroprotective response of Treg cells is mediated via IL-10 since adoptive transfer of IL-10 $^{-/-}$ T_{reg} cells fails to reduce the infarct volumes in comparison to their control mice. Therefore, it is very likely that IL-10 might be one of the factors that contribute to the neuroprotection observed under the present experimental conditions.

Another possible explanation might be due to the presence of TGF-β as several studies have shown the neuroprotective effect of TGF-β (Prehn *et al.*, 1993; Henrich-Noack *et al.*, 1996; Buisson *et al.*, 2003). There is evidence for the expression of TGF-β1 in the brain after ischemia (Krupinski *et al.*, 1996; Ruocco *et al.*, 1999; Buisson *et al.*, 2003) and it can act as a neuroprotective cytokine to limit the extent of injury (Ruocco *et al.*, 1999; Arumugam *et al.*, 2005). The administration of TGF-β1 in animal models of cerebral ischemia has demonstrated the significant reduction in the infarct volume

(Prehn *et al.*, 1993; McNeill *et al.*, 1994; Henrich-Noack *et al.*, 1996) which further supports the neuroprotective role of TGF-β. In the ischemic brain, TGF-β1 protects against the injury of the brain mediated by excitotoxic and ischemic injury (Prehn *et al.*, 1993; McNeill *et al.*, 1994; Henrich-Noack *et al.*, 1996; Arumugam *et al.*, 2005). It has also been suggested that TGF-β1 can reduce cytotoxicity mediated by microglia and deactivate macrophages (Tsunawaki *et al.*, 1988; Merrill and Zimmerman, 1991; Flanders *et al.*, 1998) thus inhibiting inflammatory responses. There are, however, other possible explanations for the observed neuroprotection. Anti-inflammatory cytokines including IL-4, IL-5 and IL-13 produce by Th-2 cells (Mosmann *et al.*, 1986; Arumugam *et al.*, 2005; Kaiko *et al.*, 2008; Wilson *et al.*, 2009) are also potential mediators which can contribute to neuroprotection. Moreover, several other studies demonstrated that different neurotrophic factors (such as BDNF, NT3 and NT4/5) and growth factors (such as TGF-β and IGF) released by lymphocytes (Kerschensteiner *et al.*, 1999; Schuhmann *et al.*, 2005; Heese *et al.*, 2006) have neuroprotective properties (McTigue *et al.*, 1998; Culmsee *et al.*, 2002; Ouyang *et al.*, 2011). Taken together, these findings suggest that these factors might have a potential role in contributing to neuroprotection under the present experimental conditions.

My second aim was to investigate whether any non-neuronal cells including astrocytes and microglia are involved in the observed neuroprotection mediated by lymphocytes. Here, I showed that the inhibition of astrocytic function using FAc, a glial cell metabolic inhibitor that has been used previously to investigate the role of astrocytic function (Swanson and Graham, 1994; Shigetomi *et al.*, 2008; Greenwood and Bushell, 2010; Henneberger *et al.*, 2010) abolished the lymphocyte-mediated neuroprotection in KA-induced cell death. This finding suggests that the observed neuroprotection is mediated via astrocytes. Supporting the current finding, several studies have reported a neuroprotective role for astrocytes (Brown, 1999; Sofroniew, 2005; Myer *et al.*, 2006). It has been shown that astrocytes stimulated by T cell-derived glutamate protect neurons from oxidative stress (Garg *et al.*, 2008). Garg *et al.* (2008) have shown that T cells produce more glutamate after activation and this signal causes metabolic modification

leading to an astrocyte-mediated neuroprotective effects. Glutamate uptake by astrocytes has been correlated with a decrease in extracellular cystine and an increase in glutathione synthesis by astrocytes (Reichelt *et al.*, 1997; Rimaniol *et al.*, 2000; Lewerenz *et al.*, 2006; Shih *et al.*, 2006), an effect that can protect neurons from oxidative stress. In addition, glutamate clearance capacity of astrocytes is enhanced by T cell-derived cytokines such as IFN-γ and IL-2 (Garg *et al.*, 2008). Moreover, astrocytes have been suggested to be involved in attenuation of microglial function (Vincent *et al.*, 1997; Hailer *et al.*, 2001; Min *et al.*, 2006) thus limiting the microglial mediated injury since microglia on activation release pro-inflammatory cytokines and reactive oxygen species which have negative role in neuronal survival (Block *et al.*, 2007; Belanger and Magistretti, 2009). Astrocytes also release various neurotrophic factors including neurotrophic growth factor (NGF), BDNF, glial-cell lined derived neurotrophic factors (GDNF), IGF and TGF-β (Blondel *et al.*, 2000; Hailer *et al.*, 2001; Farina *et al.*, 2007) along with antioxidants including glutathione and ascorbic acid that can contribute to neuroprotection against oxidative stress (Wilson, 1997; Dringen, 2000; Vargas *et al.*, 2008). However, my data showed that FAc alone was not toxic to slices and also did not induce more cell death in KA treated OSCs. This finding suggests that astrocytes are not directly involved in the modulation of KA-induced toxicity but acting as a mediator in the observed lymphocyte-mediated neuroprotection.

In contrast, in the OGD model, the inhibition of astrocytic function does not have an effect on the lymphocyte-mediated neuroprotection. A possible explanation for this result might be due to the different cell targets in OGD-induced cell death and KA-induced cell death. In stroke, not only neurons but other non-neuronal cells including astrocytes, microglia and endothelial cells are affected by ischemic injury (Doyle *et al.*, 2008) and OGD has been shown to be toxic to astrocytes in astrocyte cultures (Cao *et al.*, 2010). On the other hand, in KA-induced cell death, the majority of cells affected by KA are neurons (Schousboe *et al.*, 1992; Cheng and Sun, 1994) and it has also been reported that astrocytes were not affected by 24 hours incubation with various concentrations of KA (Waniewski and McFarland, 1990). Therefore, it is likely that

OGD might have induced astrocytic death or lowered metabolism of astrocytes under the present experimental conditions and application of glial cell metabolic inhibitor may not have any effect. Thus, these findings suggest that neuroprotection against OGD-induced cell death is mediated by soluble mediators released by lymphocytes, but astrocytes are not involved.

Nevertheless, the role of microglia in the observed neuroprotection cannot be excluded as microglial cells have also been implicated in neuroprotection (Toku *et al.*, 1998; Butovsky *et al.*, 2005; Shaked *et al.*, 2005; Polazzi and Monti, 2010). The neuroprotective role of activated microglia has been suggested via clearing cell debris (Napoli and Neumann 2009), engulfment of neutrophils (Neumann *et al.*, 2008) and release of neuroprotective factors including BDNF, IGF-1 and TGF-β (Kiefer *et al.*, 1995; Lalancette-He´bert *et al.*, 2007; Madinier *et al.*, 2009). An *in vitro* study has shown that microglial cells protect neurons from nitric-oxide induced neuronal injury (Toku *et al.*, 1998), but the mechanism of this neuroprotection is still unknown. It has further been suggested that microglial cells stimulated by INF-γ and IL-4 are neuroprotective (Butovsky *et al.*, 2005; Shaked *et al.*, 2005). However, I could not assess the role of microglia in the present study due to the lack of specific pharmacological agents to inhibit microglial function. Although, minocycline has been widely used to modulate the activity of microglia (Du *et al.*, 2001; Lin *et al.*, 2001; Wu *et al.*, 2011), minocycline has also been suggested to modulate astrocytic activity (Yrjänheikki *et al.*, 1998; Du *et al.*, 2001). Recently, FTY720 (Fingolimod), the first FDA approved oral drug for MS, has also been used to investigate its effect on microglial activity which showed apoptosis of microglia (Yoshino *et al.*, 2011) nevertheless, its effect on astrocytes has also been demonstrated (Osinde *et al.*, 2007; Choi *et al.*, 2011). Therefore, this is an important issue for future research to examine by manipulating microglial function without affecting astrocytic function.

The third aim of my study was to determine the underlying cellular signalling mechanism(s) that underlies lymphocyte-mediated response. I investigated two different

signalling pathways (MAP kinase and PI-3 kinase) which contribute in normal cellular functions, including cell differentiation, growth, cell division, survival and cell death (Rameh and Cantley, 1999; Chang and Karin, 2001; Dong *et al.*, 2002; Foster *et al.*, 2003) to determine whether these pathways underlie the lymphocyte-mediated neuroprotection. I showed that lymphocyte-mediated neuroprotection is mediated via the inhibition of ERK 1/2 and p38 MAP kinase activity without altering the effect of JNK 1/2 and, to the best of my knowledge this is the first time to show that the MAP kinase signalling pathways are involved in the lymphocyte-mediated neuroprotection. Similar findings have been previously reported by our laboratory but in PAR-2 mediated neuroprotection against KA-induced cell death in rat OSCs (Greenwood and Bushell, 2010). Normally, ERK activation is necessary for cell proliferation, cell differentiation and cell growth whereas JNK 1/2 and p38 MAP kinases are activated by stress (Xia *et al.*, 1995; English *et al.*, 1999; Chang and Karin, 2001; Dong *et al.*, 2002). It has been shown that ERKs contribute to neuronal survival by inhibiting JNK/p38 MAP kinases (Xia *et al.*, 1995) and ERK activation was suggested to be required in BDNF-mediated neuroprotection against hypoxic-ischemic injury as well as oxidative glutamate toxicity (Han and Holtzman, 2000; Rössler *et al.*, 2004). Furthermore, it has also been demonstrated that activation of ERKs was responsible for neuroprotection in neuronal culture against cytosine arabinoside (araC)-induced toxicity (Xue *et al.*, 2000). In contrast to these findings, the present data has shown that the observed neuroprotection is mediated via the down-regulation of ERK under the present experimental conditions. A possible explanation for this variation might be due to different experimental model being used as *in vivo* and *in vitro* models might respond in different ways. In addition, previous studies did not use lymphocytes and different result can therefore be expected. Another explanation might be due to the heterogeneous cell population of OSCs and it is noteworthy that OSCs contain mainly astrocytes and microglia along with neurons. Therefore, it can be suggested that the down-regulation of ERKs observed in the present study is a combined response of neuronal and non-neuronal cells and the response in pure cell preparations may vary. Meanwhile, a pharmacological approach to inhibit an MAP kinase inhibitors to mimic lymphocyte-induced reduction in MAP kinase activity

demonstrated the neuroprotection against KA-induced cell death as well as against OGD induced cell death. This finding not only suggests that the lymphocyte-induced reduction of MAP kinase activity plays a crucial role in providing neuroprotection, but also can be a potential therapeutic target for the treatment of neurodegenerative diseases. Supporting the present finding, several studies have demonstrated that inhibition of ERK activity can be neuroprotective (Alessandrini *et al.*, 1999; Satoh *et al.*, 2000; Namura *et al.*, 2001; Maddahi and Edvinsson, 2010) since persistent activation of ERKs has been found to iduce apoptosis (Chen *et al.*, 2005; Tong *et al.*, 2011). The administration of an ERK inhibitor in a stroke model of rodents significantly reduced the infract volume size thus reducing the brain damage (Alessandrini *et al.*, 1999; Maddahi and Edvinsson, 2010). One of the common mechanism of cell death in neurodegenerative diseases is oxidative stress mediated via reactive oxygen species (Pettmann and Henderson 1998) and the accumulation of reactive oxygen species has been associated with delayed and persistent ERK activation (Stanciu *et al.*, 2000). Stanciu *et al.* (2000) have further demonstrated that the persistent activation of ERK activity can be correlated with glutamate-induced toxicity in neuronal cell line and cortical neuronal cultures which was successfully reversed by U0126, an ERK inhibitor. Recently, the persistent activation of ERK has been shown in the brain of KA treated mice (De Lemos *et al.*, 2010) and zinc-induced cell death in a neuronal cell line has been suggested to be mediated via the activation of ERK (Seo *et al.*, 2001). Moreover, brain samples from stroke patients showed the upregulation of ERK activity (Slevin *et al.*, 2000) and it has also been shown that an upregulation of ERK activity after ischaemia and reperfusion contributes cell death in an animal model of stroke which was attenuated by an ERK inhibitor (Alessandrini *et al.*, 1999; Namura *et al.*, 2001). These findings therefore suggest that inhibition of ERK activity contributes neuroprotection under pathological conditions and have been a target for therapy in neurodegeneration (Cheung and Slack, 2004; Yu *et al.*, 2010). On the other hand, p38 MAP kinase activity has been reported to be activated in various neurodegenerative diseases including PD and stroke (Du *et al.*, 2001; Lin *et al.*, 2001; Nozaki *et al.*, 2001) and down-regulation of this pathway is considered to be a novel therapy in these diseases (Harper and Wilkie, 2003; Yasuda *et al.*, 2011). The activation

of p38 MAP kinases after withdrawal of growth factors in neuronal cultures has been shown to be neurotoxic which was prevented by a p38 MAP kinase inhibitor (Heidenreich and Kummer, 1996; Horstmann *et al.*, 1998). This suggests that the activation of p38 MAP kinase is neurotoxic. In addition, in an animal model of AD, the accumulation of tau proteins and release of pro-inflammatory mediators have been correlated with the activation of p38 MAP kinase leading to cell death (Savage *et al.*, 2002; Culbert *et al.*, 2006). Taken together, the present data support that down-regulation of p38 MAP kinase contributes to neuroprotection. Although, there is evidence for the involvement of JNK signalling pathways in various neurodegenerative diseases including PD and stroke contributing to cell death (Kuan and Burke, 2005; Benakis *et al.*, 2010), no alteration in JNK activity has been observed under the present experimental conditions. However, the role of JNK3 in the observed neuroprotection cannot be excluded as JNK3 has been suggested to be widely distributed in the CNS (Mohit *et al.*, 1995) and JNK3 deficiency reduces neurotrophic factors withdrawal mediated cell death in neuronal cultures (Bruckner *et al.*, 2001). Several studies have also shown the role of JNK3 in neurodegeneration (Yang *et al.*, 1997; Morishima *et al.*, 2001; Hunot *et al.*, 2004). However, the current study was unable to investigate the role of JNK3 under the present experimental conditions due to a lack of specific antibody for JNK3 and a further study with more focus on JNK3 is therefore suggested.

Next I investigated the PI-3 kinase signalling pathway to determine whether this pathway is also involved in lymphocyte-mediated neuroprotection. As I have shown that soluble factors are responsible for the observed neuroprotection and the possibility of modulation of PI-3 kinase signalling pathway by these soluble factors can not be excluded. Interestingly, the present study showed that PI-3 kinase does not appear to play a role in neuroprotection mediated by conditioned media from lymphocytes in KA-induced or OGD-induced cell death. Normally, activation of PI-3 kinase is associated with BDNF/neurotrophins-mediated neurite growth and neuritogenesis (Patapoutian and Reichardt, 2001; Tang, 2003) and several studies have demonstrated the neuroprotective role of PI-3 kinase in an *in vitro* model of stroke and in neuronal cultures (Wu *et al.*,

2004; Zamin *et al.*, 2006; Sun *et al.*, 2010). Furthermore, it has been demonstrated that the loss of dopaminergic neurons in an animal model of PD was associated with a defect in PI-3 kinase mediated signalling pathways. However, my findings are not consistent with these previous studies. A possible explanation for this inconsistency might be due the different approaches being taken by previous studies. The previous studies which showed the involvement of PI-3 kinase in neuroprotection were more focused on specific molecules like estrogen derivative (Dhandapani *et al.*, 2005) or IGF-1 (Sun *et al.*, 2010) or specific drugs such as resveratrol (Zamin *et al.*, 2006) whereas lymphocytes have been used in the present study. It is noteworthy that lymphocytes release various cytokines, chemokines and growth factors (Abbas *et al.*, 1996; Kerschensteiner *et al.*, 1999; Moalem *et al.*, 2000; Wan and Flavell, 2009) and these mediators can activate or inhibit various signalling pathways (Han and Holtzman, 2000; Zhu *et al.*, 2002; Culbert *et al.*, 2006; Lisak *et al.*, 2011). When taken together, it can be suggested that multiple signalling pathways might have been involved in the observed neuroprotection under the present experimental conditions. In addition, PI-3 kinase has also been suggested to be a key modulator in inflammatory responses (Wymann *et al.*, 2003; Haylock-Jacobs *et al.*, 2011) and has been a potential target for a drug development (Stein and Waterfield, 2000). It has been demonstrated that PI-3 kinase deficiency slows the progression of disease and improves the clinical outcomes in EAE induced mice (Berod *et al.*, 2011). However, there are different types of PI-3 kinases and their specific functions are still under investigation (Maffucci *et al.*, 2005; Williams *et al.*, 2009). Thus, more focussed research on their specific types can be suggested to investigate their precise role in lymphocyte-mediated neuroprotection.

Thus, taking all this into consideration, the present data indicate that lymphocytes including T cells and B cells are neuroprotective and similar responses have been observed in both models of neurodegenerative diseases. Furthermore, I showed that the observed neuroprotection is independent of the activation state of T cells and is also contact-independent. This shows that soluble factors released from lymphocytes are contributing neuroprotection and these factors may be one of the possible mediators to

modulate the outcome of neurodegenerative diseases. It is also noteworthy to mention that no differences were found in the response of lymphocytes and their underlying cellular signalling mechanisms between the hippocampus and the cortex of OSCs. This finding suggests that the role of lymphocytes is similar and can be considered as a single unit while developing a new therapy targeting the response of lymphocytes. However, the present study has shown a difference in the role of astrocytes in OGD-induced cell death since the inhibition of astrocytic function in the hippocampus failed to block the observed neuroprotection in the OGD model suggesting that astrocytes might have different role. I therefore suggest that astrocytes may be a good candidate to be explored while developing a new therapy for neurodegenerative diseases. However, caution should be taken while addressing the role of astrocytes. The role of microglia in neurodegenerative diseases can not be excluded as described earlier and also for the potential targets. Moreover, I also showed that lymphocyte-mediated neuroprotection underlies the inhibition of MAP kinase activity. Hence, the modulation of this signalling pathway could be a novel approach for new drug targets. I therefore believe that this information can be used to develop targeted intervention aimed to expand the knowledge of possible therapy of neurodegenerative diseases.

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Appendix

Manuscript:

1. R.Shrestha, O. Millington, J Brewer, T Bushell, "Lymphocytes exhibit neuroprotection in *in vitro* models of neurodegenerative diseases via astrocytic activation and inhibition of MAP kinase signalling pathways." (In the process of submission)

Conference presentation:

November 2011	41st Society for Neuroscience Annual Meeting (Washington DC, USA): *"Lymphocytes exhibit neuroprotection in kainate-induced cell death via astrocytic activation and inhibition of MAP kinase signalling pathways."* (Poster presentation)
July 2011	8th IBRO World Congress of Neuroscience, International Brain Research Organization (Florence, Italy): *"Lymphocytes exhibit neuroprotection in kainate-induced cell death via astrocytic activation and inhibition of MAP kinase signalling pathways."* (Poster presentation)
June 2011	5th University Research Day (Glasgow, UK): *"Immune-based therapy in neurodegenerative diseases: a translational study."* (Poster presentation)
April 2011	21st British Neuroscience Association National Meeting (Harrogate, UK): *"Lymphocytes exhibit neuroprotection in kainate-induced cell death via astrocytic activation and inhibition of MAP kinase signalling pathways."* (Poster presentation)

January 2011	2nd Glasgow Neuroscience Day (Glasgow, UK): *"Role of immune cells in central nervous system: beneficial or harmful?"* (Poster presentation)
August 2010	7th Scottish Neuroscience Group Meeting (Glasgow, UK): *"Role of T lymphocytes in the central nervous system: neuroprotective or neurotoxic?"* (Poster presentation)
June 2010	4th University Research Day (Glasgow, UK): *"Role of immune cells in central nervous system: beneficial or harmful?"* (Poster presentation)